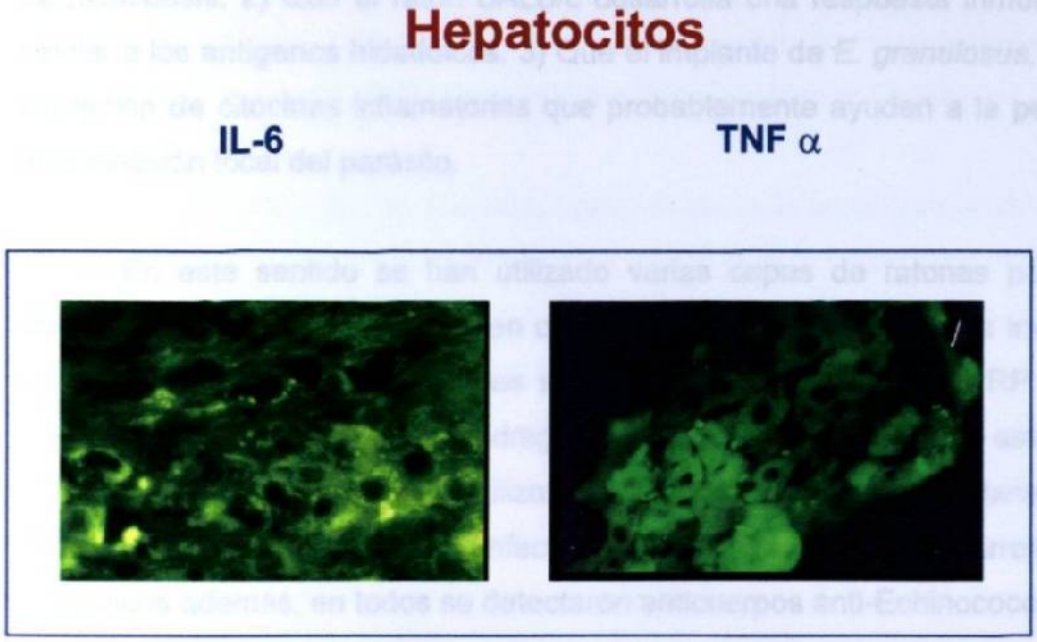

Figura 16). FISH. Secciones de hígado de ratón representativas *in situ* hibridizadas con sondas de DNA mostrando disminución de fluorescencia en células alrededor del quiste de *E. Granulosus*.

DISCUSIÓN

El presente estudio estableció un modelo experimental de hidatidosis en ratones BALB/c, para estudiar la biología de la relación huésped-parásito. Los principales resultados demuestran: 1) Que es posible establecer un modelo murino de hidatidosis. 2) Que el ratón BALB/c desarrolla una respuesta inmune humoral y celular a los antígenos hidatídicos. 3) Que él implante de *E. granulosus,* disminuye la expresión de citocinas inflamatorias que probablemente ayuden a la permanencia y diseminación local del parásito.

En este sentido se han utilizado varias cepas de ratones para inducir la enfermedad hidatídica, tomando en cuenta las experiencias de otras investigaciones en hidatidosis hepática en ratones singénicos BALB/c, (Dempser RP, *et al.* 1991, Ganguly NK, *et al.* 1986, Mondragón-de la-Peña M. C. 1995), establecimos la enfermedad hidatídica murina utilizando PSC como material infectante en ratones BALB/c, y todos los animales infectados con protoscólises, desarrollaron quistes hidatídicos además, en todos se detectaron anticuerpos anti-Echinococcus.

Una vez establecido el modelo comprobamos que el modelo murino era ideal ya que desarrollaba una respuesta inmune inicialmente normal y después estudiamos algunos mecanismos por los cuales el parásito puede evadir la respuesta inmune del huésped por medio de la inhibición de citocinas inflamatorias. Para contestar nuestra pregunta central y corroborar que el modelo animal, reconocía los diferentes epítopes del parásito, encontramos que la respuesta antígeno-específica, es manejada por dos proteínas con pesos moleculares de 60 y 31 kDa. En hidatidosis se ha descrito reactividad serológica específica a antígenos hidatídicos, (Capron A, *et al* 1967, 1968) el "arco 5" o antígeno A y (Oriol R.*et al.* 1971, Pozzuoli R *et al.* 1974. Rickard M., D. Lightowlers M. W. 1986), y otra proteína de aproximadamente ~130-150 kDa, designada como "antígeno B" (Oriol C. *et al,* 1975).

En este modelo se demostró que la infección experimental, genera los anticuerpos (Ac) contra los antígenos A y B, de 61 y 30 kDa de *Western blot* por ELISA. Se demuestra la respuesta primaria humoral entre la 2ª y 4ª semana de infección, y el "switch" ocurre hacia la 8ª semana. Otra interesante observación fue que el antígeno A dispara la respuesta inmune humoral primaria, además maneja otro antígeno de alto peso molecular ~295, esta es seguida por la respuesta secundaria contra el antígeno B. Otros antígenos con pesos moleculares con rango entre ~295 y 14 kDa también fueron reconocidos por algunos sueros.

Otra observación fue que el antígeno A (arco 5) se comportó como el epítope inmunodominante de la hidatidosis murina y que los anticuerpos IgA reconocen como único blanco hidatídico al antígeno A el cual puede ser un marcador específico de la infección activa en la hidatidosis experimental.

Con un modelo experimental de hidatidosis bien establecido, otro objetivo del trabajo fue investigar el papel de algunas citocinas en él implante de *E granulosus* en hígado, particularmente la expresión *in situ* de TNF-α e IL-6, ya que la interacción entre citocinas derivadas del huésped pueden inducir variaciones antigénicas, modificación en la virulencia, infectividad y adaptación; que son factores determinantes en la relación huésped parásito. Los principales resultados de esta investigación, indican que las citocinas inflamatorias son reguladas, negativamente disminuyendo su expresión en el hígado en respuesta a la enfermedad hidatídica.

La IL-6 es una citocina producida entre otras por células linfoides, macrófagos, fibroblastos y hepatocitos (Helle M., et al 1989), la síntesis y regulación de IL-6 puede aumentar o disminuir por diferentes antígenos, lipopolisacáridos, por TNFα, PDGF y virus durante la infección. La IL-6 tiene un importante papel como mediador. El TNFα es producido por linfocitos activados, macrófagos y células endoteliales, esto se debe a efectos pleiotrópicos, esta citocina es un importante mediador inflamatorio (Aarden L., *et al* 1985). Además el TNFα es una molécula

crítica en la resistencia contra la infección, porque tiene un profundo efecto en la inducción de IL-6 (Silacci P., *et al* 1998).

Existen reportes previos de citocinas inflamatorias en pacientes con hidatidosis hepática y han señalado un abatimiento de los niveles séricos de IL-1 y TNFα durante algunas fases de la enfermedad (Torcal J., *et al* 1996; Rigano R.,*et al* 1995, 2001); otros reportes en contraste encuentran un aumento significativo de IFN-γ, TNF-α e IL-6 en niveles séricos de pacientes con enfermedad hidatídica del hígado y pulmones, sin embargo cuando los quistes son removidos quirúrgicamente, los niveles de citocinas declinan rápidamente, estos datos experimentales nos hacen inferir que las citocinas inflamatorias juegan un papel importante, en la respuesta a *Echinococcus* (Touil-Boukoffa C., *et al* 1997). Con esta panorámica, el papel de IL-6 e TNF-α en enfermedad hidatídica pudiera ser vista como controversial, sin embargo una observación hecha por Dai & Gottstein (1999), clarifica esta discrepancia encontrando que en estados de primoinfección existe un nivel normal o aumento de transcritos de citocinas inflamatorias, sin embargo en estadios tardíos las citocinas inflamatorias son reguladas negativamente por un mecanismo dependiente de óxido nítrico, la diferencia en el comportamiento de la citocina sugiere que él implante de *Echinococcus*, restringe los efectos catabólicos crónicos producidos por el TNFα, por un mecanismo aún no definido.

Hay al menos dos vías posibles por los cuales *E granulosus* puede regular negativamente la transcripción *in situ* de TNF-α e IL-6; Primero) A traves de una inducción selectiva de citocinas Th2. Segundo) Por inhibición selectiva inducida por las hepatotoxinas producidas por el parásito. Considerando la primer posibilidad es ampliamente aceptado que la producción de citocinas Th1 disminuyen en infecciones por helmintos y por otros factores, además la producción de INFγ es inhibida por la IL-10, este balance de regulación negativa ha sido demostrado en diferentes condiciones, por lo tanto después de una agresión, la regeneración del hígado produce un aumento de IL-10, y esta citocina disminuye la producción de TNF-α (Rai R.M., *et al* 1997). (Figura 17).

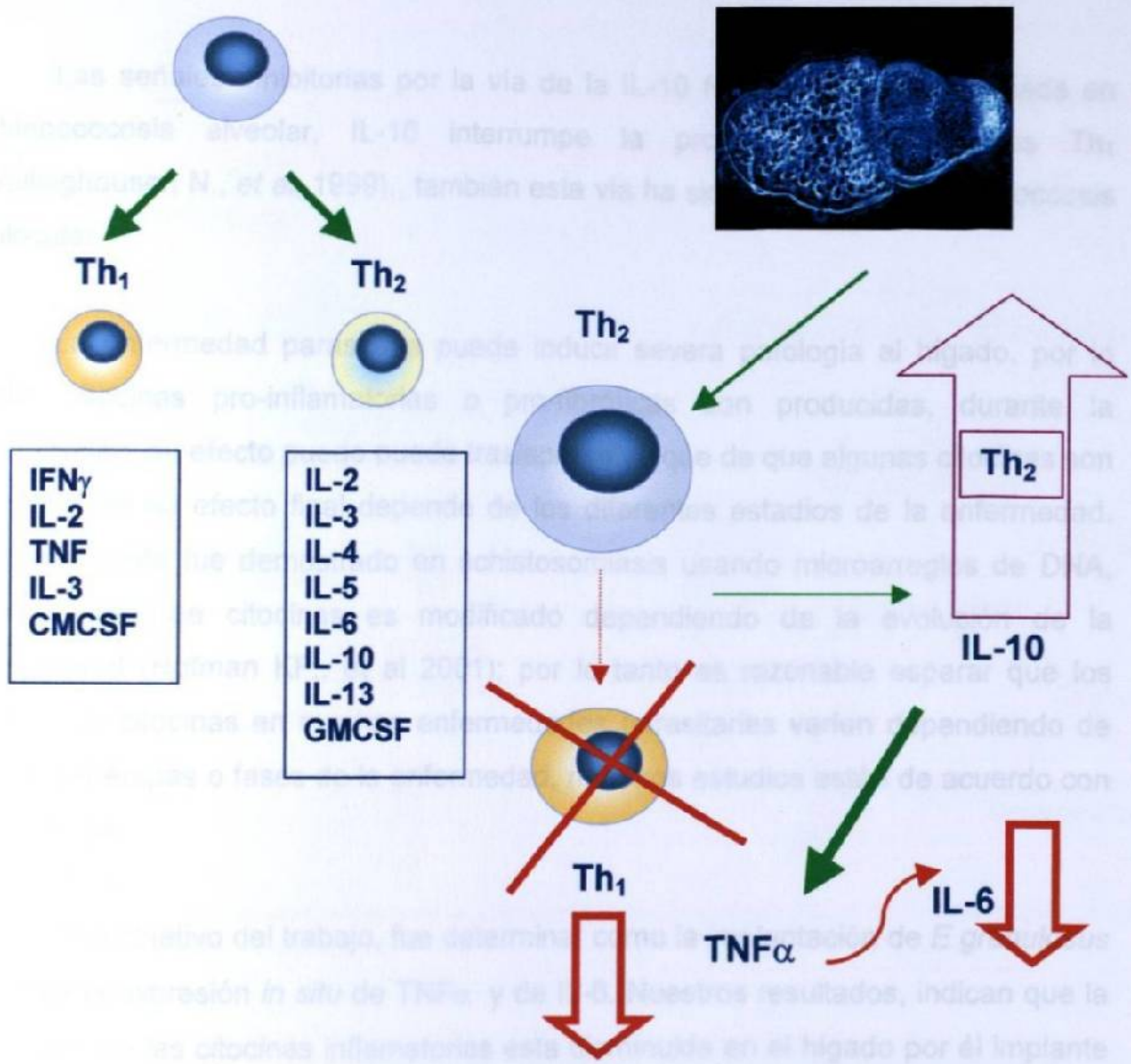

Figura 17). Representación de la expresión normal de citocinas por células Th1 y Th2, y la modificación que probablemente ocurre, al implantarse el parásito en el organismo.

Las señales inhibitorias por la vía de la IL-10 fue previamente reportada en echinococcosis alveolar, IL-10 interrumpe la producción de citocinas Th_1 (Wellinghausen N., *et al*,.1999)., también esta vía ha sido descrita en echinococcosis unilocular.

La enfermedad parasitaria puede inducir severa patología al hígado, por lo tanto, citocinas pro-inflamatorias o pro-fibróticas son producidas, durante la cicatrización, su efecto puede puede traslaparse ya que de que algunas citocinas son redundantes su efecto final depende de los diferentes estadios de la enfermedad. Recientemente fue demostrado en schistosomiasis usando microarreglos de DNA, que el perfil de citocinas es modificado dependiendo de la evolución de la enfermedad (Hofman KF., et al 2001); por lo tanto es razonable esperar que los perfiles de citocinas en muchas enfermedades parasitarias varíen dependiendo de diferentes etapas o fases de la enfermedad, nuestros estudios están de acuerdo con esta noción.

Otro objetivo del trabajo, fue determinar como la implantación de *E granulosus* modifica la expresión *in situ* de TNFα y de IL-6. Nuestros resultados, indican que la expresión de las citocinas inflamatorias esta disminuida en el hígado por él implante de *E.granulosus*, los efectos primarios de citocinas Th2 como IL-10 pudieron contribuir a esta reducción. La presencia de E. *granulosus* en el hígado puede despertar la regeneración de hepatocitos con un incremento subsecuente de IL-10, como también se puede disminuir la transcripción de TNFα (Rai. R.M. *et al*, 1997). Basados en nuestros resultados inferimos que IL-10 y TGFβ disminuyen la regulación de TNFα e IL- 6 durante la fase latente de la enfermedad (figura 18).

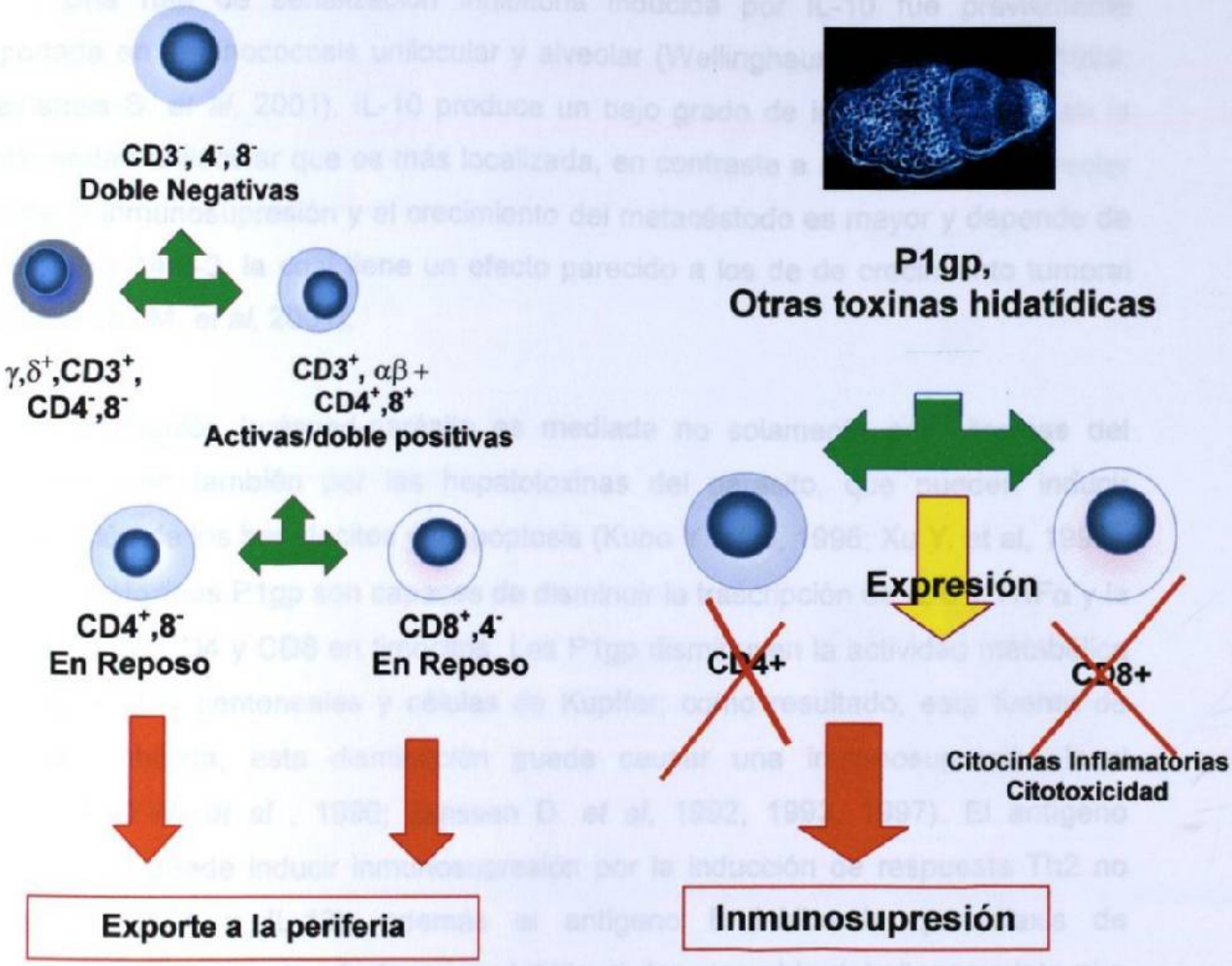

Figura 18). Representación gráfica de la ontogenia normal de células CD4 y CD8, y la probable interferencia en la expresión de CD4 y CD8 ejercida por las toxinas hidatídicas.

Una ruta de señalización inhibitoria inducida por IL-10 fue previamente reportada en equinococosis unilocular y alveolar (Wellinghausen N., *et al* P., 1999; Dematteis S. *et al*, 2001), IL-10 produce un bajo grado de inmunosupresión en la enfermedad unilocular que es más localizada, en contraste a equinococosis alveolar donde la inmunosupresión y el crecimiento del metacéstodo es mayor y depende de la proteína 14-3-3, la cual tiene un efecto parecido a los de de crecimiento tumoral (Siles-Lucas M, *et al*, 2001).

La relación huésped-parásito es mediada no solamente por citocinas del huésped, sino también por las hepatotoxinas del parásito, que pueden inducir proliferación de los hepatocitos y/o apoptosis (Kubo Y. *et al*, 1996; Xu Y. et al, 1998). Las hepatotoxinas P1gp son capaces de disminuir la trascripción de IL-6 y TNFα y la expresión de CD4 y CD8 en timocitos. Las P1gp disminuyen la actividad metabólica de macrófagos peritoneales y células de Kupffer; como resultado, esta fuente de TNFα es inhibida, esta disminución puede causar una inmunosupresión local (Acheson D.W. *et al* , 1990; Janssen D. *et al*, 1992, 1993, 1997). El antígeno hidatídico B puede inducir inmunosupresión por la inducción de respuesta Th2 no protectora (IL-4 y IL-13), además el antígeno B inhibe la quimiotaxis de polimorfonucleares, este efecto no es debido al desensamble del citoesqueleto ni a efectos tóxicos (Rigano R,. *et al*, 2001). La naturaleza de esta inhibición no esta aún determinada, sin embargo una razonable relación entre citocinas Th2 y quimiotaxis disminuida, es debido posiblemente a la baja en ciertas quimiocinas o en sus receptores, causada por IL-4, IL-10 e IL-13 (Pearlman E. *et al*, 1997; Takayama T. El al, 2001; Weber K:S:K: *et al*, 2001). (Figura 19).

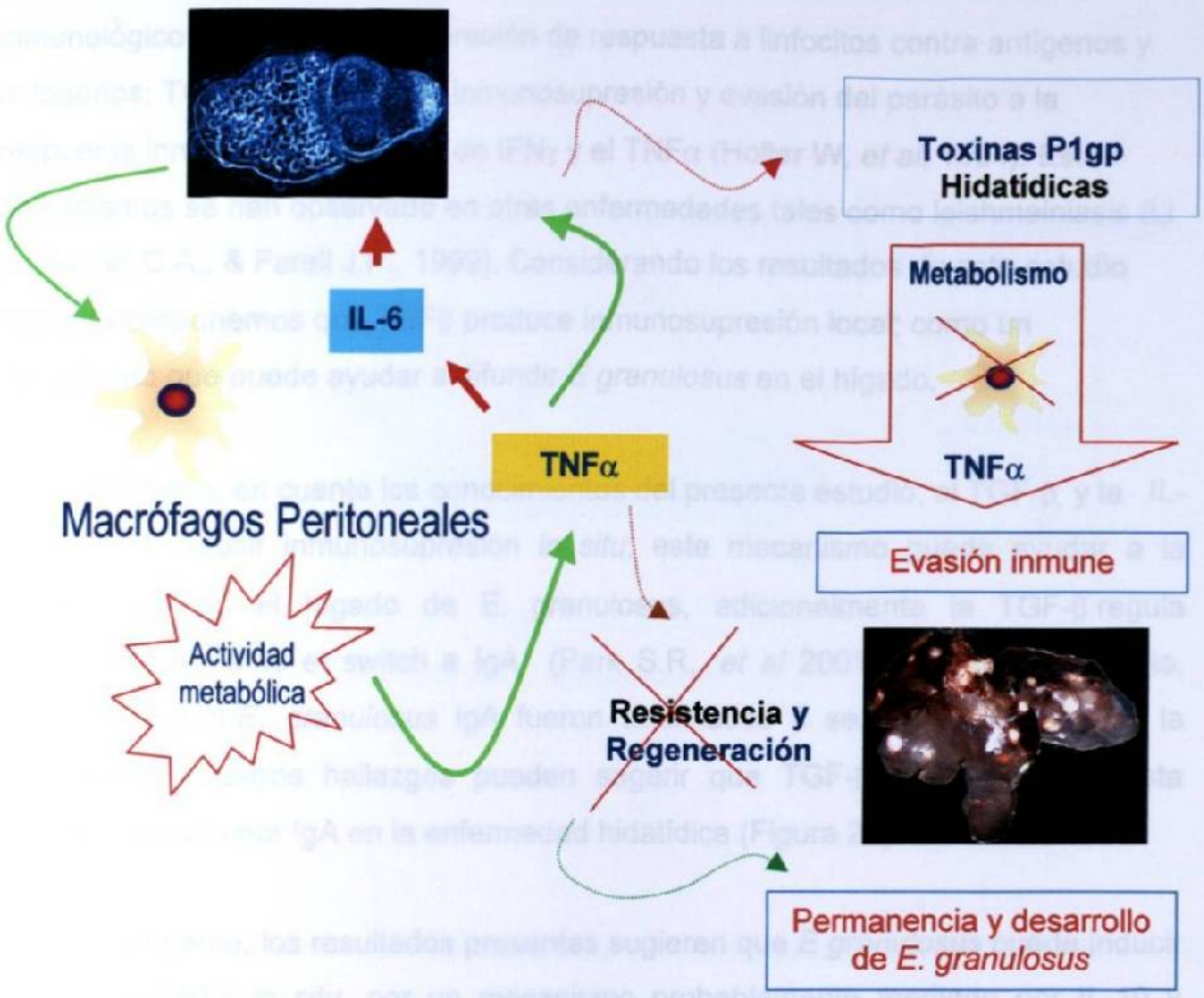

Figura 19). Representación gráfica de la producción de citocinas inflamatorias por las células de respuesta inmune al entrar el parásito en el organismo, y la probable modulación de la expresión de estas células al entrar el parásito en el organismo, y la probable modulación de la expresión de estas células al entrar las toxinas en contacto con los tejidos y así establecerse el parásito.

La citocina multifuncional TGFβ, posee una amplia variedad de efectos inmunológicos incluyendo la supresión de respuesta a linfocitos contra antígenos y mitógenos; TGFα puede inducir inmunosupresión y evasión del parásito a la respuesta inmune por inhibición de IFNγ y el TNFα (Holter W, *et al*, 1994). Estos mecanismos se han observado en otras enfermedades tales como leishmainiasis (Li J., Hunter C.A., & Farell J.P., 1999). Considerando los resultados de este estudio nosotros proponemos que TGFβ produce inmunosupresión local; como un mecanismo que puede ayudar a difundir *E granulosus* en el hígado.

Tomando en cuenta los conocimientos del presente estudio, el TGF-β, y la IL-10 pueden inducir inmunosupresión *in situ*; este mecanismo puede ayudar a la implantación en el hígado de E. granulosus, adicionalmente la TGF-β regula transcripcionalmente el switch a IgA (Park S.R., *et al* 2001), en nuestro estudio, anticuerpos anti-*E. granulosus* IgA fueron detectados 8 semanas después de la inoculación, nuestros hallazgos pueden sugerir que TGF-β regula la respuesta inmune mediada por IgA en la enfermedad hidatidica (Figura 20).

Finalmente, los resultados presentes sugieren que *E granulosus* puede inducir inmunosupresión *in situ*, por un mecanismo probablemente mediado por IL-10 y TGFα, y con estas bases podemos inferir, que el parásito escapa al daño de la respuesta inmune celular del huésped. Estas son algunas posibilidades que pueden explicar la permanencia crónica del parásito en el organismo.

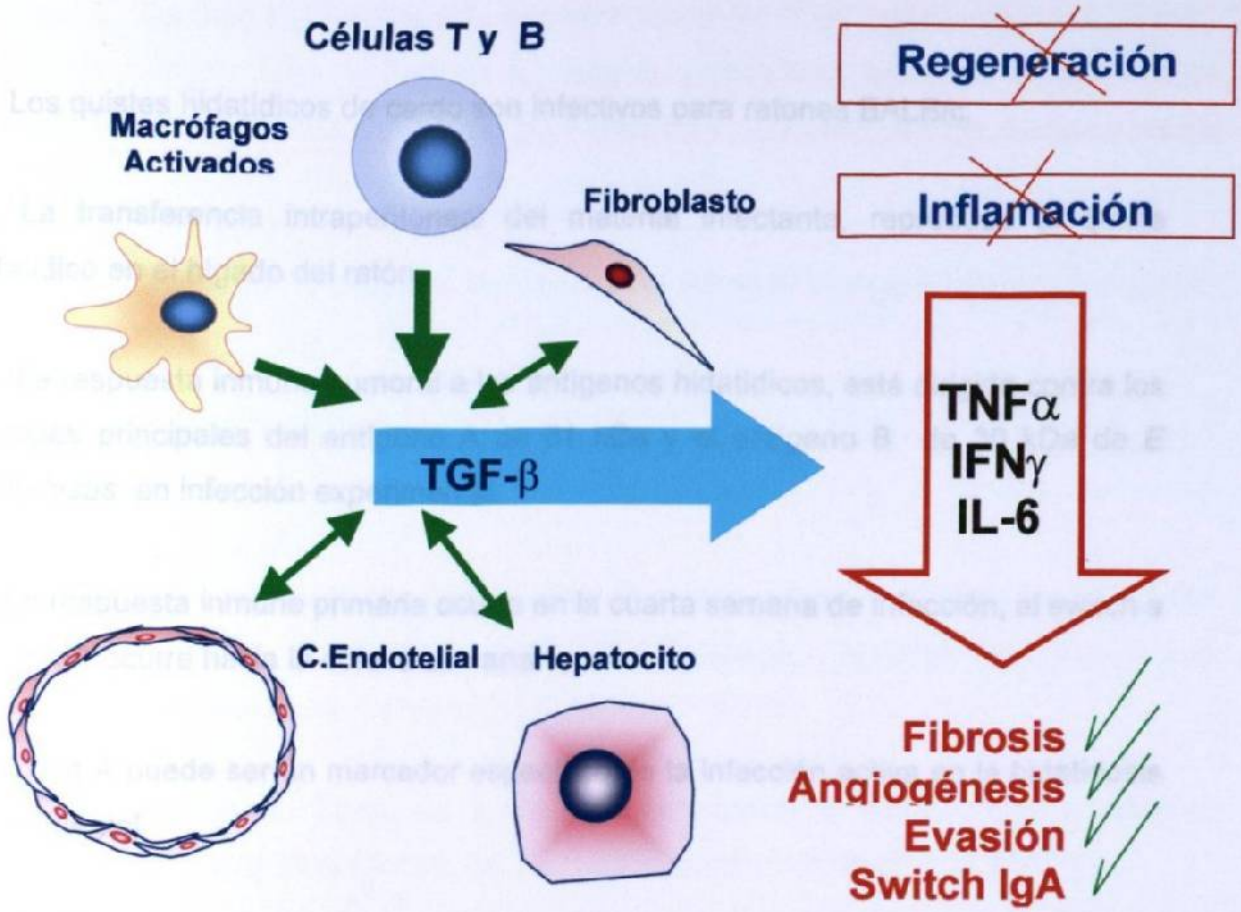

Figura 20). Representación gráfica de la producción de las diversas células, que producen TGFβ y la probable inferencia, en la modulación de la baja producción de citocinas inflamatorias, lo que facilita la implantación del parásito por largos periodos en el huésped.

CONCLUSIONES

1). Los quistes hidatídicos de cerdo son infectivos para ratones BALB/c.

2). La transferencia intraperitoneal del material infectante, reproduce el quiste hidatídico en el hígado del ratón.

3). La respuesta inmune humoral a los antígenos hidatídicos, está dirigida contra los epítopes principales del antígeno A de 61 kDa y el antígeno B de 30 kDa de *E granulosus* en infección experimental.

4). La respuesta inmune primaria ocurre en la cuarta semana de infección, el switch a IgG e IgA ocurre hacia la octava semana.

5). El Ag A puede ser un marcador específico de la infección activa en la hidatidosis experimental.

6). El implante del quiste de *E. granulosus* parece bloquear la transcripción de citocinas inflamatorias IL-6 y TNFα en hígado anulando la respuesta inmune local.

7). Son diversos los mecanismos que pueden mediar el escape del parásito como el que controla la respuesta de los genes de citocinas, los que son probablemente Inhibidos por productos del parásito .

8). La supresión de citocinas inflamatorias parece estar regulada por el TGFβ y por la IL-10.

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ECHINOCOCCUS GRANULOSUS DOWN REGULATES THE HEPATIC EXPRESSION OF INFLAMMATORY CYTOKINES IL-6 AND TNF-α IN BALB/C MICE

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Summary:

Hydatid disease is caused by the metacestode of *Echinococcus granulosus*. Different experimental models have been used to understand hydatid disease. In current studies BALB/c mice were used to evaluate the hepatic response of IL-6 and TNFα triggered by *Echinococcus granulosus*. BALB/c mice were intraperitoneally infected with protoscoleces from *E. granulosus*; hydatid cysts appeared on the liver eight weeks after inoculation. The RNA extracted from hepatic sections was used for RT-PCR amplification with primers for IL-6, TNFα, IL-10, TGFβ and G_3PDH. *In situ* cytokine expression was assessed by FISH. Complete parasite cysts on the liver surface were observed 16 weeks after infection; controls were negative. The expression of IL-6 and TNFα was normal at baseline and declined progressively eight weeks after infection; in some animals such expression was abrogated 16 weeks after infection. On the other hand IL-10 and TGFβ were increased progressively. Controls expressed the cytokines normally. Present results suggest that *E. granulosus* induces a local immunosupression probably mediated by IL-10 and TGFβ; therefore it seems possible that such a mechanism would assist the parasite in escaping the harmful host cell-mediated response.

KEY WORDS : hydatid disease, inflammatory cytokines, IL-6 mRNA, TNF-α mRNA.

Résumé : *ECHINOCOCCUS GRANULOSUS* DIMINUE L'EXPRESSION HÉPATIQUE DES CYTOKINES INFLAMMATOIRES IL-6 ET TNFα DE SOURIS BALB/C

L'hydatidose est causée par le métacestode d'Echinococcus granulosus. Différents modèles expérimentaux ont été utilisés pour comprendre cette maladie. Nous utilisons le modèle de souris BALB/c pour l'évaluation de la réaction hépatique en IL-6 et TNF-α déclenchée par Echinococcus granulosus. Les souris ont été infectées en intra-péritonéal avec des protoscolex d'E. granulosus. Après 16 semaines, la cavité abdominale a été inspectée afin de repérer le développement possible de kystes hydatidiques dans les tissus grâce à des techniques histologiques. L'ARN total a été extrait de coupes de tissus hépatiques et amplifié par la technique RT-PCR en utilisant des oligonucléotides spécifiques pour IL-6, TNF-α, IL-10, TGFβ et G_3PDH. L'expression de cytokines a été mesurée par la technique de FISH avec sondes fluorescentes d'ADN. Les kystes du parasite ont été vus à la surface hépatique 16 semaines après l'infection, tous les contrôles étant négatifs. Les cytokines inflammatoires sont apparues normalement chez les animaux non-infectés, mais l'expression de IL-6 et de TNF-α a progressivement décliné après la huitième semaine chez les animaux infectés. Chez un certain nombre de ceux-ci, les facteurs IL-6 et TNFα ont disparu dès la seizième semaine. Par contre, la présence de IL-10 et de TGFβ a progressivement augmenté. Nos résultats suggèrent que E. granulosus induit une immunosuppression locale par le biais de l'IL-10 et du TGFβ ; il est possible que par ce mécanisme, le parasite se protège des réponses immunitaires de l'organisme qui l'héberge.

MOTS CLÉS : *hydatidose, cytokines inflammatoires, IL-6 ARNm, TNF-α ARNm*

Hydatidosis is a parasitic disease caused by the metacestode (protoscoleces) from *Echinococcus* (*E. granulosus, E. multilocularis, E. oligarthrus* and *E. vogeli*), which has a world wide distribution. Infection depends on sanitary conditions in slaughters. Animal disease produces economic losses by the destruction of infected organs from affected livestock (Torgerson & Dowling, 2001; Shamesh *et al.*, 1999; Carmona *et al.*, 1999). In México, *E. granulosus* affects the porcine species and eventually human beings (Mondragón & Tavizón, 1991).

Studies in animals demonstrated: first a MHC (major histocompatibility complex) mediated immune response against a broad range of hydatid antigens (Godot *et al.*, 2000); second a cytokine mediated granulomatous reaction in different organs such as liver, lungs and other tissues. The role of cytokines has been partially studied. For example, the Th2 cytokine profile is induced by carbohydrate moieties from *E. granulosus*. Such moieties are used by the parasite to immunosupress host and spread locally. This mechanism would maintain the infection (Daemeteis *et al.*, 2001).

The parasite goes through antigenic variation by the cytokine effect, thus their virulence, infectivity and adaptation is modified (Damian, 1997). Although

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inflammatory cytokines would be increased in patient's sera with hepatic hydatidosis, a rapid decline after surgical removal is observed; in contrast, other patients show a decrease during the late phase of hydatidosis. The evident discrepancy between cytokine variations was elucidated by Dai & Gottstein (1999), who found in a murine model, normal cytokine level transcripts during early stages of infection; nevertheless they were down-regulated later by a nitric oxide-dependent mechanism, suggesting that the inflammatory cytokine profiles depend on the disease stage, in consequence Th1 cytokines seems to play a possible role against *E. granulosus* (Touil-Boukoffa *et al.*, 1997).
Our studies attempt to define the role of major inflammatory cytokines TNFα and IL-6 by implanting *E. granulosus* on murine liver.

MATERIAL AND METHODS

Protoscoleces isolation

Hydatid cysts from porcine liver were obtained by dissection. Tissues were extensively washed with PBS, fluid was aseptically collected and protoscoleces were adjusted to 2000/dose in DMEM with antibiotics (penicillin 100 U/ml, streptomycin 200 µg/ml).

Experimental infections

BALB/c mice (n = 25), were intraperitoneally infected with 2,000 protoscoleces using an insulin syringe/21 mm needle, in a 200 ml volume. Five animals/week were sacrificed at the 0, 4th, 8th, 12th, 16th weeks. Livers were examined and processed for histology, *in situ* hybridization and the RNA was extracted for RT-PCR amplification.

Reverse-transcription/polymerase chain reaction (RT-PCR)

Total RNA was extracted from several 4 µm liver sections; tissue was taken near or distant to the parasite implant. Control biopsies from healthy animals were taken from the anterior surface of the liver. RNA extraction was carried out by acid guanidium thiocyanate phenol/chloroform method (TRIzol, GIBCO-BRL). RNA was measured at 260 nm by OD. For cDNA synthesis, 250 ng of the total RNA was incubated with 200 µM dNTP and 0.7 µM of the backward primer, mixed with 5 U/20 µl of rTth/DNA polymerase (Gene Amp™ PCR system 9600). The reverse transcription was performed at 70° C for 10 min; the reaction was stopped by cooling on an ice bead. After reverse transcription, amplification of TNFα, IL-6, IL-10, TGFβ and G3PDH cDNAs was carried out by PCR by addition of 0.15 µM of the forward primer. The reaction tubes containing 50 µl of sample mixture were amplified in a thermocycler (Perkin Elmer, GeneAmp PCR system 2400), using 30 cycles under the following conditions: 94° C for two minutes, 48° C for two minutes and 72° C for 1.4 min. At the end of the PCR reaction, the samples were electrophoresed in 0.8 % agarose containing 0.5 mg/ml of ethidium bromide. PCR products were observed under UV light (Wang & Mark, 1990). An electrophoresis documentation and analysis system 120 by Kodak was used to measure the relative cytokine transcript levels by comparing the cytokine ratio: G_3PDH densitometric units for infected and non-infected animals. All controls and examined transcripts with densitometric values more than zero for calculating means. Significant differences between samples were determined by Student-t Test by Number Cruncher Statistical Systems NCSS program.

Oligonucleotides

The following oligonucleotides were used in PCR: IL-6 forward 5'-ATG AAG TTC CTC TCT GCA AGA GAC T-3', backward 5'-CAC TAG GTT TGC CGA GTA GAT CTC-3'. TNFa forward 5'-TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC-3', backward 5'-GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3', IL-10 forward 5'-CTG GAA AGA CCA AGG TGT CTA C-3', backward 5'-GAG CTG CTG CAG GAA TGA TGA-3' (Galdiero *et al.*, 1999). TGFb forward 5'-TCA CCC GCG TGC TAA TGG TGG ACC GC-3', backward 5'-ACA CCT TCC ATT CTC TTG AGC TGG G-3' (McGaha *et al.*, 2001) and G_3PDH (house keeper gene) forward 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3' and backward 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3 (Clontech).

Fluorescent *in situ* Hybridization (FISH)

Cytokines and the house-keeping mRNAs were detected in mouse liver using cDNA probes prepared by PCR as follows: a mouse library constructed in a gt11 lambda phage (Clontech, Palo Alto CA) and specific primers, were used for cDNA amplification by thermocycler, and PCR products were internally labelled with Fluoro-Green (Oligo colour kit RPN 3400 Amersham) as previously described (Fraire-Velazquez *et al.*, 1999). Tissue sections were pre-hybridized with 0.02 HCl, permeabilized with 0.01 % Triton X-100/PBS. Fluorescent probes were adjusted to 50 ng/ml of hybridization buffer/formamin (1:1), applied on tissues and incubated at 90° C for three minutes, then hybridized at 37° C for two hours, the slides were finally mounted and evaluated under epifluorescence microscopy (B-MAX 40 Olympus). Images were processed using the NIH 3 image program.

RESULTS

Animal Infections

Hydatid cysts were macroscopically observed on the liver surface eight weeks after inoculation. By the 16th week well developed cystic structures were identified; frequently two-four cysts were clumped. By microscopy, a discrete inflammatory reaction by mononuclear cells and macrophages infiltrating the hepatic tissue was observed one month after infection; the cells were organized in a granuloma. Two months after infection, a cyst with an adventitial and an incipient germinal layer was implanted along hepatic tissue. After three months, the cysts exhibited the parasite laminar and germinal membranes and the host adventitial membrane. Four months after infection, clusters of protoscoleces were evident in the germinal layer (Fig. 1). Additionally, 16 weeks after inoculation, the inflammatory reaction along implant area was decreased.

Inflammatory cytokines are expressed in the liver

All samples were normalised with the G3PDH controls. Cytokine genes were normally expressed in non-infected animals; such expression was used for baseline values. Eight weeks after infection, the IL-6 and TNFα expression decreased progressively near of parasite implant. Some animals abrogated the hepatic IL-6 and TNFα transcription 16 weeks after infection. In sharp contrast, a progressive increase of IL-10 and TGFβ was observed. On the other hand, the hepatic expression of all cytokines from a remote area of the cyst implant behaved in a similar manner to the controls. These data suggest that the parasite implant down-regulates the inflammatory cytokines (Fig. 3 and Table I).

Down-regulation of IL-6 and TNFα depends on parasite implant

To answer the question whether down-regulation was local or generalized throughout the liver, we next examined by FISH the differences in cytokine expression between sites close or distant from the cyst implant. At baseline, the mRNAs from IL-6 and TNFα were broadly detected at distant sites of the cysts; however, a remarkable decrease of these mRNA around the cyst was observed eight weeks after infection. Furthermore, the transcription was abrogated near to the implant area 16 weeks after infection. On the other hand, IL-10 and TGFβ were positive in the cyst implantation area. Non-involved tissues were faintly positive for both IL-6 and TNFα, while IL-10 and TGFβ had normal expressions. The G_3PDH house-keeping gene was positive and behaved similarity in all the tissues (Table II and Fig. 2).

DISCUSSION

The present studies were carried out to determine whether hepatic implantation of *E. granulosus* modifies *in situ* the TNFα and IL-6 expression. The main results of the current investigation indicate that inflammatory cytokines are down-regulated in the liver by *E. granulosus*; in theory, the priming effect of Th_2 cytokines such as IL-10 would contribute to this reduction. The presence of *E. granulosus* in the liver would elicit hepatocyte regeneration with a subsequent increase of IL-10; such an increase would shut-down the TNFa transcription (Rai *et al.*, 1997). Based on pre-

Cytokine	Base line	Week 4	Week 8	Week 12	Week 16
G_3PDH	393 ± 13	377 ± 12	434 ± 36	309 ± 4.9	369 ± 5.5
IL-6	373 ± 9.6	367 ± 6.9	343 ± 6.4	216 ± 22.6	4.4 ± 3.0*
TNFα	373 ± 4.1	256 ± 15	303 ± 46	16.6 ± 12	1.8 ± 3.0*
IL-10	337 ± 4.5	345 ± 33	344 ± 8.8	374 ± 5.8*	370 ± 8.8*
TGFβ	327 ± 8.0	353 ± 5.6	314 ± 10	355 ± 7.2*	367 ± 8.4*

*Significant differences with G_3PDH by Student t-Test.

Table I. – Cytokine expression in liver by RT-PCR.

Weeks of infection	IL-6 involved	IL-6 non-involved	TNFα involved	TNFα non involved	IL-10 involved	IL-10 non-involved	TGFβ involved	TGFβ non involved	G_3PDH involved	G_3PDH non-involved
0	Positive	Positive	Positive	Positive	Positive	Faint	Positive	Faint	Positive	Positive
16	Negative	Faint	Negative	Faint	Positive	Positive	Positive	Positive	Positive	Positive

Table II. – Cytokine expression in involved and non-involved hepatic tissue (FISH).

Fig. 1. – A. Protoscoleces from *E. granulosus* showing their rostellum. B. Mouse liver, one month after inoculation showing a discrete inflammatory reaction by mononuclear cells and macrophages infiltrating the hepatic tissue. Cells were organized forming a granuloma. C. Two months after infection, an incipient cyst with adventitial and germinal layer. D. Three months after infection, the cysts exhibited the parasite laminar and germinal membranes and the host adventitial layer. E. Four months after infection, the germinal layer appeared with clusters of protoscoleces.

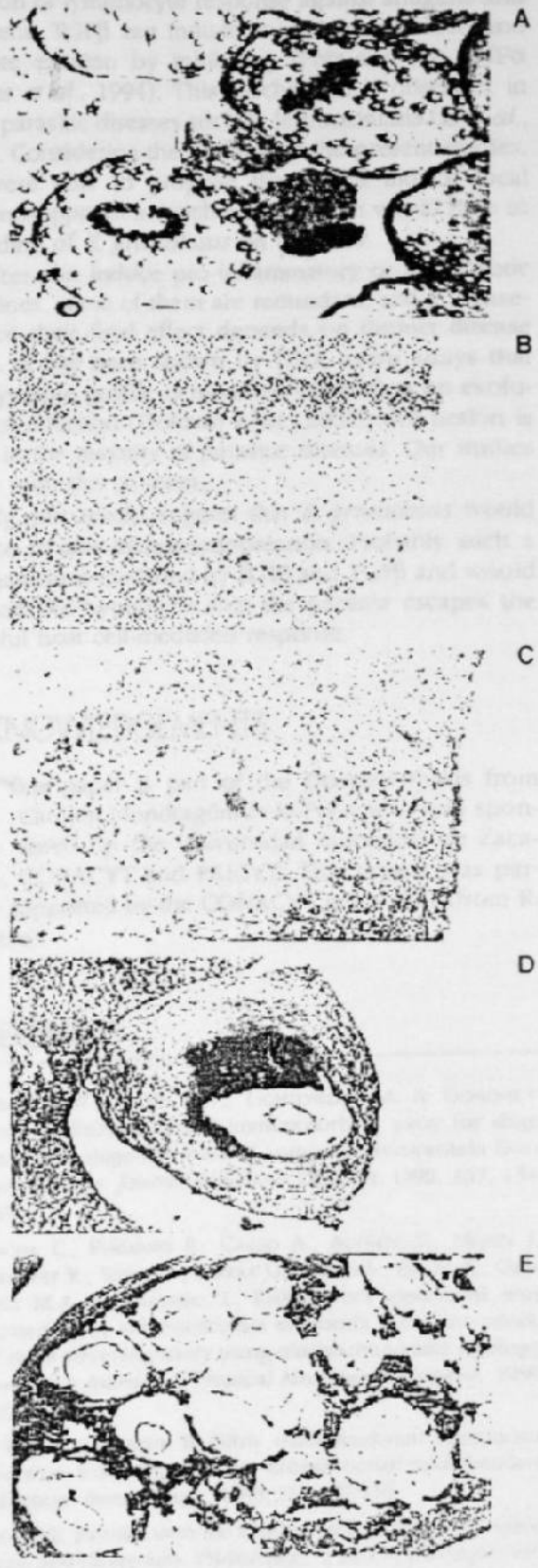
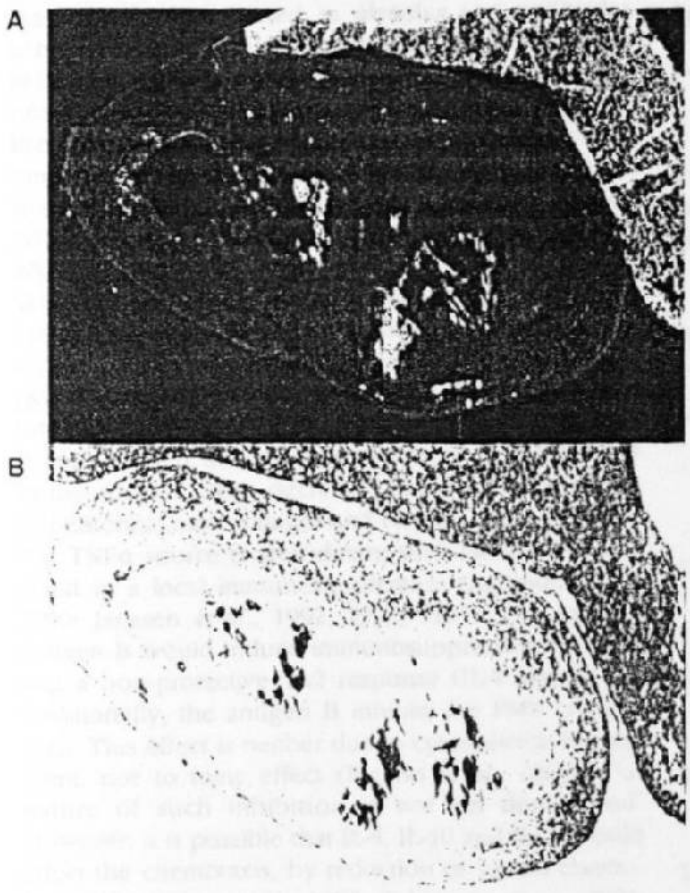


Fig. 2. – FISH. A. Representative mouse liver section *in situ* hybridized with DNA probes showing absence of mTNFα around the cyst of *E. granulosus* 16 weeks after inoculation. B. Additionally another section stained with H & E shows a poor inflammatory reaction along implant area.



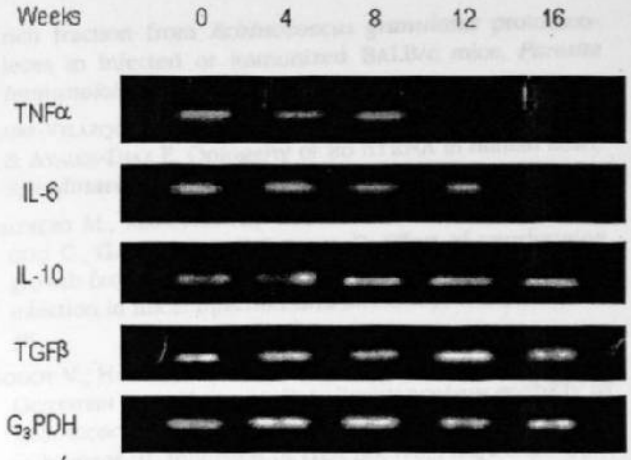


Fig. 5. – Agarose gel electrophoresis with the cytokine RT-PCR amplification products. In the bottom the G3PDH house keeping gene, above a representative panel of cytokines showing a progressive down-regulation of TNFa and IL-6 and up-regulation of IL-10 and TGFβ.

sent results, we infer that IL-10 and TGFβ down-regulate TNFα and IL-6 during the late phase of the disease. A inhibitory signaling pathway induced by the IL-10 was previously reported in alveolar and unilocular echinococcosis (Wellinghausen *et al.*, 1999; Dematteis *et al.*, 2001). IL-10 produces a lower degree of immunosuppression in unilocular disease that is more localised, in contrast to alveolar echinococcosis where the immunosuppression and the metacestode growth is higher and depends on a 14-3-3 protein which has an effect of tumor like growth factor (Sales-Lucas *et al.*, 2001).

The host-parasite relationship means not only host-cytokines; it also means parasite hepatotoxins that would induce hepatocyte proliferation or apoptosis (Kubo *et al.*, 1996; Xu *et al.*, 1998). The P1gp hepatotoxins are capable of decreasing the transcription of IL-6 and TNFα and the expression of CD4 and CD8 in thymocytes. P1gp decreases the metabolic activity of peritoneal macrophages and Kupffer cells. As result, this TNFα source is shut down; such decrease would result in a local immunesuppression (Acheson *et al.*, 1990; Janssen *et al.*, 1992, 1993, 1997). The hydatid antigen B would induce immunosuppression by eliciting a non-protective Th2 response (IL-4 and IL-13). Additionally, the antigen B inhibits the PMN chemotaxis. This effect is neither due to cytoskeleton impairment, nor to toxic effect (Rigano *et al.*, 2001). The nature of such inhibition is not yet determined. However, it is possible that IL-4, IL-10 and IL-13 would affect the chemotaxis, by reduction of certain chemokines (Pearlman *et al.*, 1997; Takayama *et al.*, 2001; Weber *et al.*, 2001).

The multifunctional cytokine TGFβ, possesses a wide variety of immunological effects including the suppression of lymphocyte response against antigens and mitogens; TGFβ can induce immunosuppression and parasite evasion by inhibiting IFNγ and the TNFα (Holter *et al.*, 1994). This mechanism is observed in other parasitic diseases such as leishmainiasis (Li *et al.*, 1999). Considering the findings of the present studies, we were able to propose that TGFβ induce local immunosuppression; such a mechanism would help to spreading of *E. granulosus* on the liver.

Parasites can induce pro-inflammatory or pro-fibrotic cytokines, some of them are redundant, and in consequence their final effect depends on distinct disease states. It has been shown by DNA micro arrays that the cytokine profile is modified depending on evolution of infection (Hofman *et al.*, 2001); this notion is valid in the majority of parasitic diseases. Our studies agree with this concept.

Finally, our results suggest that *E. granulosus* would induce *in situ* immunosuppression. Probably such a mechanism is mediated by IL-10 and TGFβ and would support the hypothesis that the parasite escapes the harmful host cell-mediated response.

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