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ECHINOCOCCUS GRANULOSUS DOWN REGULATES THE HEPATIC EXPRESSION OF INFLAMMATORY CYTOKINES IL-6 AND TNFα 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 augumenté. Nos résultats suggèrent que* E. granulosus *induit une immunosupression 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 2,000/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 µl 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 G_3PDH 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'. TNFα 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). TGFβ 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 G_3PDH 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. 2 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. 3).

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 TNFα 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	309 ± 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*	467 ± 8.4*

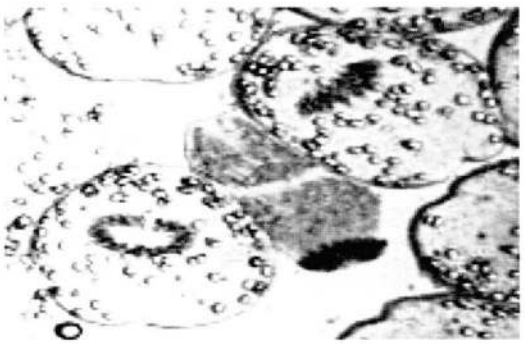
*Significant differences with G_3PDH by Student t-Test.

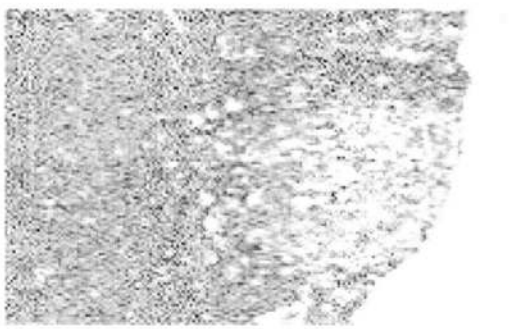
Table I. – Cytokine expression in liver by RT-PCR.

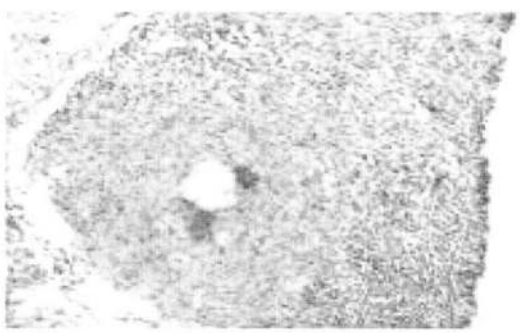
Weeks of infection	IL-6 involved	IL-6 non-involved	TNFα involved	TNFα non involved	IL-10 involved	IL-10 non-involved	TGFβ involved	TGFβ non involved	G_3PDH involved	G_3PDH non-involved
0	Positive	Positive	Positive	Positive	Positive	Faint	Positive	Faint	Positive	Positive
16	Negative	Faint	Negative	Faint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







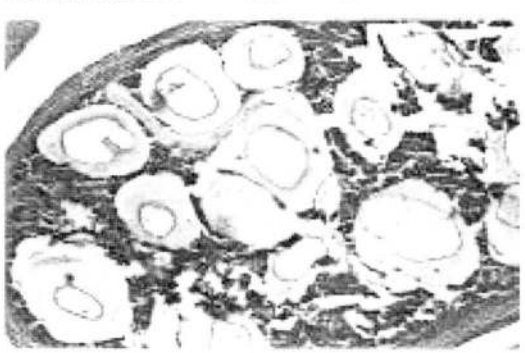
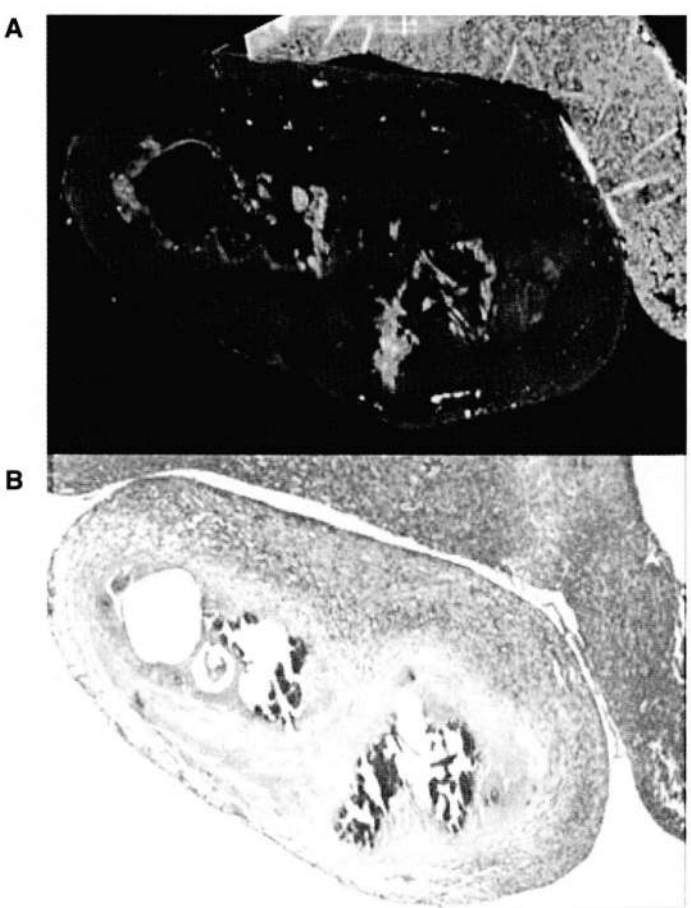


Fig. 3. – FISH. A. Representative mouse liver section *in situ* hybridized with DNA probes showing absence of mTNFα around the cyst of *E. granulosus* 16 weeks after inoculation. B. Additionally another section stained with H & E shows a poor inflammatory reaction along implant area.



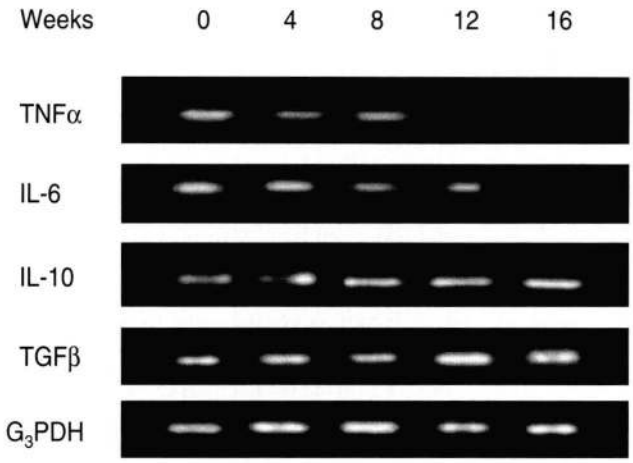


Fig. 2. – Agarose gel electrophoresis with the cytokine RT-PCR amplification products. In the bottom the G_3PDH house keeping gene, above a representative panel of cytokines showing a progressive down-regulation of TNFa and IL-6 and up-regulation of IL-10 and TGFβ.

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 (Siles-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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