Echinococcus granulosus Down Regulates the Hepatic Expression of Inflammatory Cytokines IL-6 and TNFα in BALB/c Mice


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 protoscolecies 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. 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, TNFα 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 protoscolecies 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 GpPDH. 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 TNFα a progressivement diminué 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, TNFα ARNm.

Hydatidosis is a parasitic disease caused by the metacestode (protoscolecies) 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 immunosuppress 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
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 (Toil-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 (Geneset 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.

Oligonucleotides

The following oligonucleotides were used in PCR: IL-6 forward 5’-ATG AAG TTC TCT TGT GGA AGA GAC T-3’, backward 5’-CAG TAG GTT TGC CGA GTA GAT CTC-3’. TNFα forward 5’-TTC TGT CTA CTG GTA GAC CAC CCG GTG ATC GGT CC-3’, backward 5’-GTA GAT AGC AAA TCG GCT GAC GGT GTG GG-3’, IL-10 forward 5’-CTG GAA AGA CCA AGG TGG TCT CTA C-3’, backward 5’-GAG CTG CTG CAG GAA TGA TGA-3’ (Galdiero et al., 1999). TGFβ forward 5’-TCA CCC GGG TGC TAA TGG ACC GC-3’, backward 5’-ACA CCT TCC ATT CTC TTG AGC TGG G-3’ (McGaha et al., 2001) and G3PDH (house keeper gene) forward 5’-TGA AGG TGG TGA ACG GAT TTG GC-3’ and backward 5’-CAT GTA GGC CAT GAG TGT 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. 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 G3PDH 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 Th1 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-

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Base line</th>
<th>Week 4</th>
<th>Week 8</th>
<th>Week 12</th>
<th>Week 16</th>
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<tr>
<td>G3PDH</td>
<td>393 ± 13</td>
<td>377 ± 12</td>
<td>434 ± 36</td>
<td>309 ± 4.9</td>
<td>309 ± 5.5</td>
</tr>
<tr>
<td>IL-6</td>
<td>373 ± 9.6</td>
<td>367 ± 6.9</td>
<td>343 ± 6.4</td>
<td>216 ± 22.6</td>
<td>4.4 ± 3.0*</td>
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<tr>
<td>TNFα</td>
<td>373 ± 4.1</td>
<td>256 ± 15</td>
<td>303 ± 46</td>
<td>16.6 ± 12</td>
<td>1.8 ± 3.0*</td>
</tr>
<tr>
<td>IL-10</td>
<td>337 ± 4.5</td>
<td>345 ± 33</td>
<td>344 ± 8.8</td>
<td>374 ± 5.8*</td>
<td>370 ± 8.8*</td>
</tr>
<tr>
<td>TGFβ</td>
<td>327 ± 8.0</td>
<td>353 ± 5.6</td>
<td>314 ± 10</td>
<td>355 ± 7.2*</td>
<td>467 ± 8.4*</td>
</tr>
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*Significant differences with G3PDH by Student t-Test.

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

<table>
<thead>
<tr>
<th>Weeks of infection</th>
<th>IL-6 non-involved</th>
<th>TNFα non-involved</th>
<th>IL-10 non-involved</th>
<th>TGFβ non-involved</th>
<th>G3PDH non-involved</th>
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<tr>
<td>0</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
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<tr>
<td>16</td>
<td>Negative</td>
<td>Faint</td>
<td>Positive</td>
<td>Faint</td>
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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.
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 leishmaniasis (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 stages. 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.

ACKNOWLEDGEMENTS

This paper is part of the Doctoral thesis from Carmen Mondragón-de-la-Peña who was sponsored by: the Universidad Autónoma de Zacatecas, CONACYT and PAICYT. This project was partially supported by the CONACYT grant 1877 (from R. Herrera).

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Fig. 2. - 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 TNFα and IL-6 and up-regulation of IL-10 and TGFβ.
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