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Echinococcus multilocularis: Inflammatory and regulatory chemokine responses in patients with progressive, stable and cured alveolar echinococcosis

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Index Descriptors and Abbreviations:
AE, alveolar echinococcosis
CCL, CC chemokine ligand
CCR, CC chemokine receptor
GRO-α, growth related oncogene, alpha
LARC, liver and activation regulated chemokine
MCP-3, monocyte chemoattractant protein 3
MCP-4, monocyte chemoattractant protein 4
MDC, macrophage derived chemokine
MIG, monokine induced by interferon gamma
MIP-1α, macrophage inflammatory chemokine, alpha
MIP-1β, macrophage inflammatory chemokine, beta
PARC, pulmonary and activation regulated chemokine
RANTES, regulated on activation, normally T cell expressed and secreted
TARC, thymus and activation regulated chemokine
Cestode
Parasite infection
Echinococcus multilocularis
Fox tape worm
Alveolar echinococcosis
Echinococcosis patients
State of infection
Immune response
Chemokine
Inflammation

ABSTRACT

The progressive growth of *Echinococcus multilocularis* metacestodes and their tissue infiltration will cause organ malfunction and finally failure. In few patients, *E. multilocularis* metacestode proliferation will spontaneously regress, but little is known about the determinants which may restrain metacestode survival and growth. In this study, chemokine responses were investigated in *E. multilocularis* patients at different states of infection, i.e. with progressive, stable and cured alveolar echinococcosis (AE). Characteristic chemokine profiles and changes in their production were observed in AE patients and infection-free controls when their peripheral blood cells were cultured with *E. multilocularis* antigens. The production of CC and CXC chemokines which associate with inflammation (MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5 and GRO-α/CXCL1) was constitutively larger in AE patients than in controls; and the elevated chemokine releases were equal in patients with progressive, stable or cured AE. Cluster analyses identified three distinct chemokine response profiles; chemokines were enhanced, depressed or produced in similar quantities in AE patients and controls. A disparate cellular responsiveness was observed in AE patients to viable *E. multilocularis* vesicles; cluster 1 (GRO-α/CXCL1, MCP-3/CCL7, MCP-4/CCL13, TARC/CCL17, LARC/CCL20) and cluster 2 chemokines (PARC/CCL18, MDC/CCL22, MIG/CXCL9) were clearly diminished, while cluster 3 chemokines (MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5) augmented. The increased production of inflammatory chemokines in patients even with cured AE could be induced by residual *E. multilocularis* metacestode lesions which continuously stimulate production of inflammatory chemokines. *E. multilocularis* metacestodes also suppressed cellular chemokine production in AE patients, and this may constitute an immune escape mechanism which reduces inflammatory host responses, prevents tissue destruction and organ damage, but may also facilitate parasite persistence.

1. Introduction

Human alveolar echinococcosis (AE), caused by the larval metacestode stage of *Echinococcus multilocularis*, has a high mortality rate in untreated patients. The progressive tumor–like growth of

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metacestodes, and their tissue-infiltrating proliferation will cause organ malfunction and finally failure. *E. multilocularis* metacestodes will shed off proglotid- and germinal cell-containing vesicles (Hemphill and Gottstein, 1995) which disseminate into various body sites where they establish and proliferate into secondary metacestode parasite lesions. Early diagnosis of AE is difficult as symptoms of infection will not appear until the parasite has caused severe damage in the infected organs by mass proliferation and local or regional "metastasis"; and such AE is often diagnosed only years post infection (Gottstein and Hemphill, 1997). In most AE patients, a complete cure is not possible, even after radical surgical resection of the metacestodes, and the available chemotherapy only inhibits metacestode growth but will not kill the parasite (Kern et al., 2003).

A few patients have been identified in whom *E. multilocularis* metacestode lesions were found calcified (Gottstein and Felleisen, 1995), and this raises the possibility that protective immune responses against parasite growth and dissemination may exist. But little is known about the determinants which may restrain metacestode survival and proliferation, or eliminate *E. multilocularis* infection. In patients with abortive versus progressive AE, an opposite cytokine secretion profile was observed with dominant interleukin (IL) -10, IL-5 and interferon (IFN)-γ associated with progressive AE (Godot et al., 2000), while in experimental studies, relative resistance to *E. multilocularis* growth was attributed to pronounced and sustained cellular proliferation and high levels of Th1 type cytokines (Emery et al., 1997). The pronounced granulomatous cellular infiltrates around viable as well as calcified metacestodes suggest an involvement of effector cells which may add to control metacestode growth and proliferation. Chemokines account for monocyte, granulocyte and lymphocyte dissemination, directed cellular migration, cell adhesion, tissue infiltration and cell activation, and such, these chemotactic cytokines may promote host resistance by activating immune functions that kill, expel, or sequester *E. multilocularis*. In the present study, we have investigated the cellular chemokine responses in AE patients with progressive, stable and cured *E. multilocularis* metacestode lesions, and compared them with infection-free controls. Disparate chemokine profiles were observed; in patients infected with *E. multilocularis* pro-inflammatory cellular chemokine responses prevailed while regulatory and anti-inflammatory chemokines were depressed.

2. Subjects, materials and methods

2.1. Study participants

A total of 72 AE patients, who visited the echinococcosis consultation at the University Hospitals of Ulm, were recruited. Blood samples of 10 healthy controls were received from the University Hospitals of Tübingen. The AE patients and infection-free controls came from south-western Germany (Baden-Württemberg and Bayern). The Echinococcosis Centre and University Clinics of Ulm and the Clinics of Tübingen are situated in an endemic area for *E. multilocularis*, both towns being 70 km apart. The mean age of AE patients was 55 years, ranging from 21 to 87 years. 57% of the patients were female. The AE patient group contained 11 cured patients, 39 patients had stable infections and 22 patients had progressive AE. Diagnosis of AE was achieved by positive imaging, serology and histology, and most patients were re-examined regularly during follow-up. All patients gave written consent to participate in this study and approval for the investigation was obtained from the ethical board of the University Clinics of Ulm (Ethikkommission Antrag No. 71/2004). All patients with progressive or stable AE except seven were treated with benzimidazole (albendazole or mebendazole).

2.2. In vitro culture of E. multilocularis metacestodes

In vitro culture of *E. multilocularis* metacestodes was carried out as described previously (Dreweck et al., 1999; Hemphill et al., 2003). Briefly, metacestode tissues were removed aseptically from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) and cut into tissue blocks. These were cultivated in complete medium (as below in 2.4) at 37 °C, saturated humidity and 5% CO_2. Several weeks later metacestodes started to proliferate and to produce daughter vesicles. Once a week the culture medium was renewed and the supernatant of *E. multilocularis* metacestode cultures (EmMed = *E. multilocularis* metacestode culture medium) and viable *E. multilocularis* metacestode vesicles (EmVes = *E. multilocularis* metacestode vesicles) were collected and used immediately for in vitro stimulation of PBMC.

2.3. Preparation of antigens

PHA and LPS were purchased from Sigma, St Louis, MO, USA. *Ascaris lumbricoides* antigen (AscAg) was prepared as described previously (Diallo et al., 2004). For *E. multilocularis* vesicle fluid antigen (EmVF) preparation, *E. multilocularis* vesicles were homogenized and sonicated (30% intensity, pulse 1 s for 8 min) on ice. The vesicle homogenate was centrifuged at 5000g for 30 min at 4 °C. The supernatant was sterile-filtrated (0.22 μm) and stored at −70 °C. For the generation of the *E. multilocularis* metacestode tissue antigen (EmMet), solid metacestode tissues were grinded with a Ten-Broek tissue grinder on ice until a homogenous liquid extract was produced; then, this extract was centrifuged at 15,000g for 30 min at 4 °C, the liquid supernatant was sterile-filtered (0.22 μm) and stored at −70 °C. The protein concentration of each *E. multilocularis* preparation as well as of the *A. lumbricoides* antigen was determined by the bicinchoninic acid (BCA) protein determination kit (Pierce, Rockford, IL, USA).

2.4. Isolation of peripheral blood mononuclear cells (PBMC)

Whole blood samples of donor blood from AE patients were processed at the blood transfusion centre at the University Hospitals of Ulm, buffy coated cells of healthy controls were processed at the blood transfusion centre at the University Hospitals of Tübingen. PBMC from AE patients and healthy endemic controls were isolated by Ficoll-Paque (Pharmacia, Freiburg, Germany) density gradient centrifugation. Cell culture experiments were conducted as described previously (Eger et al., 2003). Briefly, PBMC were adjusted to 1×10^7 cells/ml in complete medium: RPMI-1640 containing 25 mM Hepes, 2 mM L-glutamine (Gibco, Eching, Germany), 10% heat-inactivated fetal calf serum (FCS) (Biochrom KG, Berlin, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma, St. Louis, MO, USA). PBMC (0.2×10^7) were plated in 96-well flat-bottomed tissue culture plates (Costar 3548, New York, USA) and cultured in the presence of either 50 μl MedCon (complete medium), 50 μl EmMed (supernatant of *E. multilocularis* metacestode cultures), 50 μl EmVes (2–3 viable *E. multilocularis* metacestode vesicles washed and transferred in complete medium), 50 μl EmVF (*E. multilocularis* vesicle fluid antigen in complete medium, final concentration 60 μg/ml), 50 μl EmMet (*E. multilocularis* metacestode antigen in complete medium, final concentration 60 μg/ml), 50 μl LPS (*Escherichia coli* Lipopolysaccharide; 026:B6; Sigma; in complete medium, final concentration 6 μg/ml), 50 μl PHA (phytohaemagglutinin in complete medium, final concentration 5 μg/ml) (Sigma), or 50 μl AscAg (*Ascaris lumbricoides* antigen in complete medium, final concentration 120 μg/ml) and incubated for 48 h at 37 °C, saturated humidity and 5% CO_2.