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Molecular survival strategies of *Echinococcus multilocularis* in the murine host

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Abstract

Larval infection with *Echinococcus multilocularis* starts with the intrahepatic postoncospheral development of a metacestode that—at its mature stage—consists of an inner germinal and an outer laminated layer (GL & LL). In certain cases, an appropriate host immune response may inhibit parasite proliferation. Several lines of evidence obtained in vivo and in vitro indicate the important bio-protective role of the LL. For instance, the LL has been proposed to protect the GL from nitric oxide produced by periparasitic macrophages and dendritic cells, and also to prevent immune recognition by surrounding T cells. On the other hand, the high periparasitic NO production by peritoneal exsudate cells contributes to periparasitic immunosuppression, explaining why iNOS deficienct mice exhibit a significantly lower susceptibility towards experimental infection. The intense periparasitic granulomatous infiltration indicates a strong host–parasite interaction, and the involvement of cellular immunity in control of the metacestode growth kinetics is strongly suggested by experiments carried out in T cell deficient mouse strains. Carbohydrate components of the LL, such as Em2(G11) and Em492, as well as other parasite metabolites yield immunomodulatory effects that allow the parasite to survive in the host. I.e., the IgG response to the Em2(G11)-antigen takes place independently of alpha-beta+CD4+T cells, and in the absence of interactions between CD40 and CD40 ligand. Such parasite molecules also interfere with antigen presentation and cell activation, leading to a mixed Th1/Th2-type response at the later stage of infection. Furthermore, Em492 and other (not yet published) purified parasite metabolites suppress ConA and antigen-stimulated splenocyte proliferation. Infected mouse macrophages (AE-MØ) as antigen presenting cells (APC) exhibited a reduced ability to present a conventional antigen (chicken ovalbumin, C-Ova) to specific responder lymph node T cells when compared to normal MØ. As AE-MØ fully maintain their capacity to appropriately process antigens, a failure in T cell receptor occupancy by antigen-Ia complex or/and altered co-stimulatory signals can be excluded. Studying the status of accessory molecules implicated in T cell stimulation by MØ, it could be shown that B7-1 (CD80) and B7-2 (CD86) remained unchanged, whereas CD40 was down-regulated and CD54 (=ICAM-1) slightly up-regulated. FACS analysis of peritoneal cells revealed a decrease in the percentage of CD4+ and CD8+T cells in AE-infected mice. Taken together the obstructed presenting-activity of AE-MØ appeared to trigger an unresponsiveness of T cells leading to the suppression of their clonal expansion during the chronic phase of AE infection. Interesting information on the parasite survival strategy and potential can be obtained upon in vitro and in vivo treatment. Hence, we provided very innovative results by showing that nitazoxanide, and now also, respectively, new modified compounds may represent a useful alternative to albendazole. In the context of chemotherapeutical repression of parasite growth, we searched also for parasite molecules, whose expression levels correlate with the viability and growth activity of *E. multilocularis* metacestode. Expression levels of *14-3-3* and *II/3-10*, relatively quantified by realtime reverse transcription-PCR using a housekeeping gene *beta-actin*, were studied in permissive nu/nu and in low-permissive wild type BALB/c mice. At 2 months p.i., the transcription level of *14-3-3* was significantly higher in parasites actively proliferating in nu/nu mice compared to parasites moderately growing in wild type mice. Immunoblotting experiments confirmed at the protein level that 14-3-3 was over-expressed in parasites derived from nu/nu mice at 2 months p.i. In vitro-treatment of *E. multilocularis* with an anti-echinococcal drug nitazoxanide for a period of 8 days resulted in a significant decrease of

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both *14-3-3* and *II/3-10* transcription levels, which correlated with the kinetics of a housekeeping gene, *beta-actin*. This indicates that 14-3-3- exhibits a good potential as a molecular marker to assess viability and growth activity of the parasite.

Keywords: *Echinococcus multilocularis*; Mouse; T cell; Macrophage; Nitric oxide; Immunosuppression; Nitazoxanide

1. Biologically active molecules from *Echinococcus multilocularis* metacestodes

Excreted/secreted parasite molecules play a crucial role in the host parasite relationship, especially in view of elucidating potential survival mechanisms of *Echinococcus multilocularis*, such as inducing anergy in the host immune response or modulation of the immune response towards ineffectiveness. We hypothesize that the process of chronic infection is divided into different phases, and thus at different time points different modulatory metabolites are expressed.

By affinity chromatography of *E. multilocularis* extracts on a CNBr-activated Sepharose-coupled mAbE492/G1, we have isolated a carbohydrate-rich fraction named Em492-antigen, which suppresses ConA-triggered as well as *E. multilocularis* antigen-induced splenocyte proliferation [1]. To investigated whether the protein- or the carbohydrate-part of Em492 antigen would account for the inhibition of splenocyte proliferation, we treated the purified Em492 antigen either with proteinase K in order to enzymatically remove the protein part, or with 50 mM sodium periodate to chemically degrade carbohydrate residues. We found, that chemical degradation of carbohydrate residues did dramatically abolish the proliferation inhibitory effect, while treatment with proteinase K did not affect the activity of Em492 antigen. This suggests that the activity associated with inhibition of splenocyte proliferation can be attributed to carbohydrates, rather than to proteinous components of Em492 antigen.

Previous studies, involving viability tests and electron microscopy, have shown that simple cellular cytotoxicity does not account for Em492 activity [1]. Two other potential options how Em492 could act on splenocytes were investigated. First, Em492 could modulate nitric oxide production of macrophages, which could have adverse effects on splenocyte proliferation. Second, Em492 could act directly on T cells and contribute to T cell apoptosis, and by this effect cause diminished spleen cell proliferation. For studies on macrophages, exsudate cells (PEC) from naive and 2 month infected BALB/c mice were collected, and adherent, macrophage-enriched, cell populations were isolated. They were stimulated with LPS in the presence or absence of different concentrations of Em492 antigen. After 48 h nitrite production was hardly detectable in control and Em492-treated macrophages, nitrite was clearly enhanced upon LPS-stimulation. However, nitrite accumulation was doubled in supernatants of peritoneal macrophages co-incubated with LPS plus Em492-antigen. This suggests that Em492 antigen enhanced the LPS-mediated production of nitric oxide, which in turn could be responsible for the inhibition of proliferation in spleen cell populations. For studies on T cell apoptosis, A1.1 T hybridoma cells were used. Cells were preincubated with medium containing Em492 antigen for 1 h, and were then transferred to tissue culture plates coated with anti-CD3 (anti-mouse CD3, 2C11 clone) antibodies for 24 h to induce apoptosis. Annexin V staining was performed and cells were assessed by FACS analysis. A combination of Em492 antigen preincubation and subsequent anti-CD3 treatment resulted in 70% of the cell population positive for annexin V, while anti-CD3 alone caused 50% of the cell population to undergo apoptosis. In controls (no treatments or Em492 antigen treatment without anti-CD3) 20% of T cell were exhibiting apoptotic features. This suggests that Em492 could contribute to increased apoptosis in certain lymphoid cells in the periparasitic region.

2. Addressing immunosuppression in experimental murine AE

An impairment of the accessory function of macrophages originating from mice infected with *E. multilocularis* (AE-MØ) was described already some time ago [2,3]. Further to these studies, we carried out experiments to confirm the defective accessory-molecule-expression by AE-MØ and its respective consequence in T cell activation [4]. In a first experiment, we examined whether mice immunized by intraperitoneal injection of chicken ovalbumin (C-Ova) can provide immunocompetent infiltrating peritoneal macrophages that express co-stimulator molecules. FACS analysis showed essentially an up-regulation of B7 receptors, which are the main receptors, implicated in T cells activation. These MØ were used as a positive control regarding the expression of co-stimulatory molecules. A second experimental approach consisted in blocking co-stimulatory molecules (B7-1, B7-2) and CD40 with appropriate antibodies. These molecules were blocked on potentially immunocompetent naïve macrophages cultivated either with naïve peritoneal T cells or peritoneal T cells isolated from AE-infected mice in the presence of ConA. Results demonstrated that the neutralization of B7-1 (CD80) and B7-2 (CD86) simultaneously reduced peritoneal naïve T cell and AE-T cell proliferation, thus revealing the importance of B7 receptor expression within the process of T cell stimulation. The reduction phenomenon was cell number dependent. The treatment of naïve macrophages with anti-CD40 increased ConA-driven T cell proliferation. This high T cell response can be explained by the implication of a B7 co-stimulator, which is up-regulated following signalization of naïve macrophages with anti-CD40, this according to findings obtained by other authors [5].

3. The *E. multilocularis* 14-3-3-protein family

Members of the 14-3-3-protein family have been identified as regulatory molecules in intracellular signaling pathways and cell cycle control [6–8]. Previously, the first *Echinococcus* 14-