Studies in a co-infection murine model of Plasmodium chabaudi chabaudi and Leishmania infantum: interferon-γ and interleukin-4 mRNA expression


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This work aimed to study the T helper type 1/2 (Th 1/Th2) cytokine profile in a co-infection murine model of Plasmodium chabaudi chabaudi and Leishmania infantum. Expression of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) was analyzed, in spleen and liver of C57BL/6 mice, by reverse transcriptase-polymerase chain reaction. High levels of IFN-γ expression did not prevent the progression of Leishmania in co-infected mice and Leishmania infection did not interfere with the Th1/Th2 switch necessary for Plasmodium control. The presence of IL-4 at day 28 in co-infected mice, essential for Plasmodium elimination, was probably a key factor on the exacerbation of the Leishmania infection.

Key words: co-infection - Plasmodium - Leishmania

Malaria and leishmaniasis are currently two of the major causes of mortality and morbidity among human parasitic infections, with an enormous social and economic impact. Human cases of concomitant malaria, Plasmodium falciparum and leishmaniasis, Leishmania donovani have been reported (Nandy et al. 1995, Sah et al. 2002).

In 1980s, Coleman and his colleagues developed several studies of interactions between L. mexicana and P. yoelii in the murine model, that demonstrated an elevation of severity and susceptibility to the diseases during co-infection. However, they did not investigate the immunological mechanisms involved (Coleman et al. 1988a, b, c). The P. chabaudi chabaudi/L. infantum murine model could reveal parasite-specific immune responses and mechanisms of regulation of T-cell subsets during a concomitant infection. Regulation of immune response by CD4+ T helper (Th) cells and cytokine products have in fact been shown to be essential during both Plasmodium and Leishmania infections.

P. c. chabaudi strain AS infection, in C57BL/6 mice strain, induces a sequential immune response by the two major subsets of CD4+ Th cells (Langhorne 1989). The acute primary parasitaemia, with a peak level around day 7, is resolved by the Th1 type response and related cytokines, mainly interferon-gamma (IFN-γ) (Langhorne 1989, Zhong & Stevenson 2000). The control and clearance of chronic subpatent blood infection during the second phase of infection are related with the Th2 pattern of cytokine production, namely interleukin-4 (IL-4), that is crucial for B cells maturation and subsequent antibody release (Langhorne 1989, Taylor-Robinson & Phillips 1994).

The murine malaria P. c. chabaudi is a recognized model for analyzing the acquired immunity to the asexual erythrocytic stages of malaria parasites especially for P. falciparum infections of humans (reviewed by Taylor-Robinson 1998).

In murine models of leishmaniasis, it is widely assumed that resistance to the disease is correlated with the production of IFN-γ by Th1 cells, while susceptibility is associated with Th2 response, resulting in IL-4 production (Heinzel et al. 1989, Gumy et al. 2004), although in visceral leishmaniasis this clear-cut role of Th1/Th2 is less evident (Honore et al. 1998).

The main goals of this study were to evaluate whether the initial immune responses to P. c. chabaudi could be blocked, when mice were co-infected by L. infantum, thus preventing the Th1/Th2 switch, and to investigate if resistance or susceptibility to L. infantum could be modulated by a previous activation of Th1 cells. In order to clarify these questions, we analysed mRNA expression of IFN-γ and IL-4, both in spleen and liver of co-infected C57BL/6 mice, by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

C57BL/6 female mice, 5 to 8 weeks old were purchased from Harlam Interfauna Ibérica SL (Barcelona, Spain) and kept in the IHMT animal facilities, according to the European Union requirements (86/609/CEE) recognized in Portuguese law (DR DL129/92 and Portaria 1005/92). P. c. chabaudi AS was obtained from the Institute of Cell, Animal and Population Biology, University of Edinburgh and maintained by continuous blood passage. L. infantum MON-1 amastigotes were isolated from spleen and liver of infected golden hamsters (Mesocricetus auratus), according to Cenini et al. (1989).

Mice were divided into the following groups: (i) intrauterinal inoculation with isotonic saline solution, (ii) intrauterinal inoculation with 10⁶ P. c. chabaudi AS.
parasitised red blood cells (pRBC), (iii) intraperontional inoculation with $10^6$ L. infantum (MON-1) amastigotes, and (iv) intraperontional inoculation of $10^6$ P. c. chabaudi AS pRBC, and seven days later with $10^6$ L. infantum (MON-1) amastigotes.

Three mice of each group were sacrificed by cervical dislocation and fragments of spleen and liver were collected at days 0 (P. c. chabaudi inoculation day), 7 (L. infantum inoculation day), 14, 28, 35, and 56.

DNA extraction from sample tissue (spleen and liver) was carried out according to Boom et al. (1990) and PCR amplification for the diagnosis of infection caused by L. infantum was performed using primers based on a complete DNA sequence of L. infantum kinetoplast minicircle (Cortes et al. 2004).

L. infantum load was monitored by determination of parasite density in spleen and liver of groups 3 and 4, using the limiting dilution method (Buffet et al. 1995).

P. c. chabaudi parasitaemia was monitored and determined during the course of infection of groups 2 and 4 by optical microscopic observation of thin blood smears made from mice tail snips and stained with Giemsa.

Fragments of spleen and liver collected for RNA extraction were stored in liquid nitrogen until use. Extraction of total RNA was performed with TRIzol® reagent (Invitrogen-Life Technologies, New York, US), according to the manufacturer’s protocol. Synthesis and amplification of cDNA were performed as described by Centeno-Lima et al. (2002). Specific primers for murine IFN-γ, IL-4 and hypoxanthine-guanine phosphoribosyl transferase (HPRT) as an internal standard were used (Centeno-Lima et al. 2002).

After electrophoresis on 1% agarose gel, DNA was transferred to a positively charged nylon membrane by common blotting procedures. Blots were hybridised with a specific digoxigenin (DIG) labelled oligoprobe for each cytokine and HPRT as previously described by Centeno-Lima et al. (2002). The oligonucleotide probe-labelling protocol was performed according to the manufacturer’s protocol (Roche Applied Science). Hybridization chemiluminescent signals were quantified and converted to optical density units (OD) using Phoretix 1D plus software (Phoretix International). Results were normalized for the relative quantity of cytokine mRNA (arbitrary units) by dividing the OD of the cytokine by the corresponding OD of HPRT, for each sample. Data analysis was carried out using Statistical Package for Social Sciences 11.0 Program (SPSS®). The non-parametric Mann-Whitney rank sum test was used to assess statistical significance for values of $P < 0.05$. The results of the following groups were statistically compared at days 14, 28, 35, and 56 after P. c. chabaudi infection: group 2 vs group 4 and group 3 vs group 4.

RESULTS

Mice infected with P. c. chabaudi alone (group 2) showed an expected peak of parasitaemia at day 7 (37%) that was followed by a drastic reduction of parasitaemia. Two recrudescences were noted at days 16 and 30 before total eradication of blood parasites, verified by optical microscopy observation (Fig. 1).

All cultures from the spleen and liver samples, collected in mice inoculated with L. infantum only (group 3) were negative throughout the experiment, however the presence of Leishmania DNA was confirmed in both organs by PCR.

The course of blood stage P. c. chabaudi infection in co-infected mice (group 4) was similar to what was described for group 2, though mice from group 4 reached higher levels of parasitaemia until day 14, exhibiting recrudescence at days 16, 26, and 30 (Fig. 1).

Spleen from co-infected mice showed positive promastigote cultures at day 56, with a parasite density mean value of $8 \times 10^4$ parasites per g of organ, as determined by the limiting dilution method. The high Leishmaniana parasite density observed at day 56 was accompanied by an evident splenomegaly on all three mice.

The IFN-γ expression in the spleen and liver of mice infected with P. c. chabaudi only, reached a peak at day 7 that coincided with peak of parasitaemia. The expression level of IFN-γ decreased afterwards (Fig. 2a, b).

Co-infected mice showed elevated IFN-γ expression at day 56, both in the spleen and liver, and significant differences were noted in the liver between this group and mice infected P. c. chabaudi only (Fig. 2a, b).

In mice infected with L. infantum only the IFN-γ expression levels were generally lower than in co-infected mice and in spleen, IFN-γ expression was only detectable at day 28. Significant differences between group 3 and group 4 were observed in the liver at days 14 and 56 (Fig. 2a, b).

The IL-4 gene expression was detected in the spleen of groups 2 and 4, with a peak at day 28. At this day, the IL-4 expression levels were significantly higher in co-infected mice than in Leishmania infected mice (Fig. 2c). This cytokine was not detected in the liver of mice from any group (data not showed).
Despite the existence of overlapping endemic areas, scarce information is known on concomitant malaria and leishmaniasis and no references are available regarding cytokine analysis in mixed infections involving *Plasmodium* and *Leishmania*. In the present study, the analysis of cytokine mRNA expression in spleen and liver cells during the early phase of erythrocytic *P. c. chabaudi* infection, confirmed that in C57BL/6, there is a Th1 inflammatory response, associated with high expression of IFN-γ by day 7 post-infection (peak of parasitaemia) in order to control parasitaemia (Langhorne 1989, Zhong & Stevenson 2000). In parallel, our results confirmed a progressive elevation of IL-4 expression, a Th2 cytokine characteristic of the chronic stage of *P. c. chabaudi* infection, responsible for B cells maturation and antibody release, which are necessary for recrudescence control and total elimination of blood parasites (Langhorne 1989, Taylor-Robinson & Phillips 1994). When all *Plasmodium* parasites are eradicated from the blood (around day 35), the IL-4 expression becomes undetectable, both in plasmodium-only and co-infected mice.

Although the course of parasitaemia was similar in mice infected only with *P. c. chabaudi* and in co-infected mice, the IFN-γ expression level seemed to persist from day 14 onwards in the co-infected group, mainly in the spleen, and then markedly increased at day 56. This sustained IFN-γ expression may be related to the fact that on the day of *Leishmania* inoculation, a Th1 immune response had already been established as a result of the *Plasmodium* infection. Although some authors point out to the importance of IFN-γ in the resolution of leishmaniasis infection (Kaye et al. 1991, Carvalho et al. 1994), the IFN-γ expression levels detected at day 7, when *Leishmania* parasites were inoculated, apparently did not restrain the progression of *L. infantum* infection in co-infected mice. According to Melby et al. (2001), syrian hamsters infected with *L. donovani* are not able to control parasite replication despite the high levels of Th1 cytokine expression, namely IFN-γ, as observed in this study. In fact, co-infected mice presented an evident splenomegaly on day 56, which is a typical clinical symptom of visceral leishmaniasis, and positive cultures with high values of parasite density were observed. In addition, there was no detectable erythrocytic *Plasmodium* parasites. Concomitant *Plasmodium* infection seems to increase the susceptibility to *Leishmania*, as there were no clinical signs or positive cultures from mice infected with *Leishmania* only.

*L. infantum* infection had little effect on the Th1/Th2 switch necessary for *P. c. chabaudi* control. The differences on IFN-γ expression observed at day 14 in co-infected group were not sufficient to interfere with *P. c. chabaudi* parasitaemia as it was probably already under control. On the other hand, the presence of IFN-γ at the time of *Leishmania* infection might have determined the high levels of IFN-γ observed at day 56. Himmelrich et al. (2000) mention that exogenous IL-4 administration to C57BL/6 mice does not modify their resistant phenotype nor redirect effector cells to a Th2 phenotype. However, the presence of IL-4 at day 28, essential for *P. c. chabaudi* elimination, was probably a key factor on the outcome of *Leishmania* infection (IL-4 was not detected in *Leishmania* infected only) leading to an increase in the *Leishmania* parasite load at day 56 associated with a high IFN-γ production. The higher expression levels of IL-4 on the day 28 might have been determinant, as it probably disturbed the fine tune of the mixed Th1/Th2 response observed in *L. infantum* infected mice (Honoré et al. 1998).

On the other hand, we can not exclude the importance
of other cytokines during this concomitant parasitic infection. For example, a high expression and production of IL-10 as well as IFN-γ were detected in dogs experimentally infected with L. infantum (Santos-Gomes et al. 2002) and by day 28 and 56 after infection with L. donovani, there was a high splenic and hepatic mRNA expression of IL-10 and TGF-β in hamsters (Melby et al. 2001). Belkaid et al. (2002) also showed that the persistence of parasites of L. major in C57BL/6 is related with the production of IL-10 by CD4+CD25+ regulatory T cells.

In conclusion, a fine tuning between Th1 and Th2 type responses is essential for the resolution of these parasitic infections. However the co-infection, that often occurs in nature, can disrupt this balance, leading to disease exacerbation.

Although this study comes across as a preliminary investigation, to our knowledge, is the first work in the laboratory, in order to explore and firmly define the impact of co-infection on Th1/Th2 immune responses.

REFERENCES


