Detection of clinical-stage specific molecular *Toxoplasma gondii* gene patterns in patients with toxoplasmic lymphadenitis

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Three cases of symptomatic toxoplasmic lymphadenitis, together with a serologic profile of recent infection, are described, for which quantitative real-time PCR (LightCycler PCR) targeting different parasite genes was designed, in order to quantify *Toxoplasma gondii* DNA in acute and follow-up blood specimens. Similar parasite gene kinetics and DNA concentrations were observed in the patients studied. However, the profile of each target gene investigated was different. While the level of B1 DNA remained elevated for the entire time of observation, irrespective of clinical and serologic resolution, the SAG-1 gene was detected at the end of acute symptomatic disease, overlapping with a strong anti-*T. gondii* IgA antibody response, and persisting for over 3 months after infection and clinical recovery. With respect to the two bradyzoite genes investigated (SAG-4 and MAG-1), levels peaked during the symptomatic phase, but did not fall until 2 or 3 months of follow up. The real-time PCR assay with new alternative targets to the B1 gene may have potential for monitoring the clinical outcome of disease and for providing molecular information regarding the actual state of infection.

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INTRODUCTION

Toxoplasmosis is a widespread zoonosis, caused by the protozoan *Toxoplasma gondii*. In the immunocompetent host, the infection is most often asymptomatic and self-limiting, and lymphadenopathy is the most common clinical manifestation of the infection (Durlach *et al.*, 2003). Serologic testing is the initial and primary method of diagnosis. Acute infection is likely if an elevated IgM antibody titre is present. In patients who have equivocal IgM antibody results (after 3 months), detection of IgA or IgE antibodies may be helpful (Montoya & Remington, 1995). Because the functional affinity of specific IgG antibodies is initially low and increases during subsequent weeks, the measurement of high-avidity antibodies may help to discriminate between recently acquired and old infection (Montoya *et al.*, 2004).

The use of PCR to diagnose *T. gondii* lymphadenitis is unnecessary, although it is occasionally performed on biopsy material. In suspect serologic cases or when the clinical diagnosis is doubtful, PCR-based techniques could be performed on clinical specimens. Most PCR-based techniques make use of the B1 gene, and less commonly the SAG-1 (P-30) single-copy sequence, which has been shown to be a satisfactory PCR target for the detection of *T. gondii*. Other single-copy sequences, including the SAG-2, SAG-3, GRA-4

and ROP-1 genes, have been used as PCR targets in research laboratories. Recently, PCR and real-time PCR technology with LightCycler, SYBR Green or TaqMan chemistry have been employed for the molecular detection of the parasite and stage differentiation (Costa *et al.*, 2000; Reischl *et al.*, 2003; Hierl *et al.*, 2004; Contini *et al.*, 2005; Kupferschmidt *et al.*, 2001) in a variety of patients and clinical settings.

In order to quantify *T. gondii* DNA during acute and convalescent disease and to understand the molecular details of *T. gondii* stage conversion, we designed and developed a new real-time fluorescence PCR with the LightCycler protocol, using primers targeting multiple tachyzoite and bradyzoite stage-specific genes, and evaluated the assay with acute and follow-up blood specimens from three immunocompetent patients with lymphadenitis.

METHODS

From February 2004 to November 2005, polymorphonuclear blood monocyte cell (PBMC) samples were collected from three patients with symptomatic toxoplasmic lymphadenitis (two males, one female, mean age 27 ± 4.5 years). Four or five PBMC specimens were taken from each patient. These subjects presented at the Unit of Infectious Diseases of the University of Ferrara, because of a 1- or 2-week history of single or multiple adenomegalies, headache and generalized weakness and fever. The duration of lymphadenopathy was 34 ± 1.2 days. The immunological response was compatible with acute toxoplasma infection. Anti-toxoplasma antibodies (Vidas; bioMérieux) showed high IgM (4.58 ± 3.7 IU ml⁻¹), IgA (1.1 ± 6.5 IU ml⁻¹) and

Abbreviations: LC-PCR, LightCycler PCR; PBMC, polymorphonuclear blood monocyte cell.

IgG (102.4 ± 4.8 IU ml⁻¹) levels, with a low Toxo IgG avidity (Vidas, Toxo IgG Avidity; bioMérieux) value ($3.9 \pm 1.2\%$) at the time of diagnosis; the Remington test (IgM-IFA) was positive in all cases. Patients underwent PBMC collection (one sample) at the time of presentation of clinical symptoms; two follow-up specimens were taken at day 14 or 30 after the onset of lymphadenopathy, and two further PBMC samples were taken during the asymptomatic period (60 and 90 days). Owing to her acute discomfort, one patient (female, not pregnant) was empirically treated for 2 weeks with TMP-SMX (960 mg *per os*), which led to complete resolution of constitutional symptoms and lymphadenopathy.

PBMC specimens were isolated by density-gradient centrifugation over Fycoll-paque plus (Amersham Biosciences Europe). DNA was extracted from PBMCs, within 2 h of collection, and examined by a real-time LightCycler PCR (LC-PCR) assay, which used the LC FastStart DNA Master SYBR Green I technology (Roche Molecular Biochemicals), as previously described (Contini *et al.*, 2005). The primer pairs employed amplified the 35-fold repeated B1 gene of *T. gondii* (Burg *et al.*, 1989), the SAG-1 gene that encodes tachyzoite surface antigen P-30 (Savva *et al.*, 1990), and the SAG-4 and MAG-1 genes that encode the bradyzoite 18 kDa surface protein and the 65 kDa cyst-surface antigen, respectively (Odeberg-Ferragut *et al.*, 1996; Parmley *et al.*, 1994). Gene amplification and LC-PCR conditions were those described previously (Contini *et al.*, 2002, 2005). The analytical sensitivity of our LC-PCR had previously been evaluated by a 10-fold dilution series of *T. gondii*-positive plasmid clones in duplicate to construct a standard curve. Also, the specificity of the primers was repeatedly tested with PBMC DNA specimens from immunocompetent persons without serological evidence of previous toxoplasma exposure, matched with the above cases for age, sex and demographics, as described previously (Contini *et al.*, 2005).

To quantitate and monitor parasite DNA load in PBMC specimens, the crossing point (Cp) at which the fluorescence of a given sample significantly exceeded the baseline signal was calculated. The results were expressed as a fractional cycle number. The obtained Cp values were plotted against the known concentration of the parasite to establish a standard curve. The parasite count for a given PBMC sample was calculated by interpolation from this curve (Lightcycler software version 3.5). According to earlier reports (Costa *et al.*, 2000), for SAG-1 gene amplification, the sensitivity of the system is such that a single parasite can be distinguished from background (one parasite corresponds to 0.1 pg DNA). A similar sensitivity is obtained for the MAG-1 and SAG-4 genes. For the B1 gene, the assay was able to estimate the *T. gondii* DNA concentration down to 10⁻³ parasites per millilitre (data not shown).

RESULTS AND DISCUSSION

In Fig. 1 are shown the parasite gene kinetics observed during 3 months of observation. *T. gondii* kinetics were similar for each of the patients examined. MAG-1 DNA appeared at the time of diagnosis, but fell 14 days later. After a second peak at 30 days (end of symptomatic period), this gene decreased significantly and was undetectable after the second month (convalescent phase). SAG-4 DNA concentrations began to rise at the sixth day, peaked between 14 and 30 days, continued to be elevated to 60 days, but began to fall significantly at the end of the second month. *T. gondii* B1 DNA, which rose at the beginning of the symptomatic infection with a slight reduction at day 14, had a significant increase of DNA copy number at 30 days, and remained elevated for over 90 days of observation, irrespective of symptom and serologic resolution. Table 1 shows the different *T. gondii* DNA levels at different times. The patient under specific treatment did not show detectable B1 DNA levels in samples collected after a few days of therapy (data not shown), thus confirming previous observations (Contini *et al.*, 2002; Foudrinier *et al.*, 1996). With respect to the SAG-1 profile and the monitoring of parasite load in the PBMCs, the DNA concentration was measured at very low levels at the time of diagnosis, reached a peak at 30 days, and remained elevated at a similar level in all patients for the remainder of the study (Table 1, Fig. 2a, b).

The conversion between tachyzoite and bradyzoite forms is rapid with respect to antibody secretion, and is promoted by factors which are not completely known. The virulence of the parasite strain and host factors also have to be taken into account.

In vitro studies have suggested that bradyzoite antigens are expressed before cyst formation (Gross *et al.*, 1996). This may explain the simultaneous detection of MAG-1 and SAG-4 genes together with B1, before the appearance of the SAG-1 gene. SAG-1 (the so-called P-30 antigen) is the major stage-specific surface antigen of the *T. gondii* tachyzoite, but not of bradyzoites or tissue cysts, and it is also an important parasite ligand involved in the process of attachment to host

Fig. 1. *T. gondii* gene kinetics monitored with real-time LC-PCR in PBMCs from patients with lymphadenitis during 3 months of observation. ●, SAG-4; ■, MAG-1; ▲, B1; ◆, SAG-1.

Table 1. Monitoring of *T. gondii* DNA load concentrations evaluated with real-time LC-PCR at different times of infection from initial diagnosis

The *T. gondii* SAG-1 DNA began to be detected in large amounts during the third week and persisted in the convalescent period. Statistical comparisons between the different groups were analysed by using the non-parametric Mann–Whitney *U* test; significant difference at $P < 0.05$.

<i>T. gondii</i> gene	No. of patient PBMC specimens	Parasites ml ⁻¹ (mean ± SEM)		
		Time of diagnosis (day 0)	Symptomatic infection (14–30 days)	Convalescent phase (60–90 days)
B1	5	$1.62 \times 10^2 \pm 0.81 \times 10^2$	0.59 ± 0.1 ; $21.2 \pm 6.9^*$	0.81 ± 0.25 ; $16.83 \times 10^2 \pm 0.92 \times 10^2$
SAG-1	4	0.03 ± 0.19	3.8 ± 1.1 ; $4.5 \times 10^2 \pm 2.3 \times 10^2 \dagger$	3.2 ± 1.6 ; 3.4×10^2
SAG-4	5	99 ± 1.45 ; 108 ± 0.21	130 ± 1.1 ; $136 \times 10^2 \pm 0.28 \times 10^2 \ddagger$	0.81 ± 0.01 ; $0.96 \times 10^2 \pm 0.14 \times 10^2$
MAG-1	5	$0.033 \times 10^2 \pm 0.011 \times 10^2$	0.004 ± 0.0 ; $0.07 \times 10^2 \pm 0.24 \times 10^2$	Negative

* $P < 0.001$ compared to 0, 14 and 60 day samples; $P < 0.05$ compared to 90 days.

† $P < 0.001$ and $P < 0.05$ compared to 0 and 14 days, respectively; $P < 0.01$ and $P > 0.5$ compared to 60 and 90 day samples, respectively.

‡ $P < 0.001$ compared to 0, 14 and 60 day samples; $P < 0.05$ compared to 90 days.

cells (Mineo & Kasper Lloyd, 1994). It elicits high IgA mucosal levels, which may be undetectable in early acute infection and at the end of infection.

In this regard, the highest levels of blood SAG-1 DNA were always detected at the end of acute symptomatic disease, and overlapped in the third week with a strong IgA anti-*Toxoplasma* serological response that persisted for 3 months following infection. Despite this potent immune response, SAG-1 continued to be detectable in clinical blood specimens

at all sample points in the study, in the absence of clinical signs of disease or relapse. It is likely that tachyzoites express distinct sets of surface antigens, which may contribute to an immune-evasion mechanism that allows toxoplasmas to persist (Kim & Boothroyd, 2005).

The significance of PCR amplification from bradyzoites in clinical samples also needs to be further explored. The SAG-4 and MAG-1 genes showed kinetics that in part mirrored previous observations in patients with toxoplasmic

Fig. 2. (a) SAG-1 gene standard curve performed by real-time SYBR Green LC-PCR. Amplification plots were obtained for *T. gondii* DNA 10-fold dilutions from 10^7 to 1 parasites ml⁻¹. Each slope corresponds to a particular input target quantity. (b) Monitoring of parasite load in PBMCs at the time of diagnosis (specimen 1), and during symptomatic (specimens 2 and 3) and asymptomatic periods (specimen 4). The *T. gondii* SAG-1 DNA gene began to be detected in large amounts during the third week (4.5×10^2 parasites ml⁻¹) and persisted in the convalescent period (eighth week, specimen 4, parasite amount detectable = 3.4×10^2 parasites ml⁻¹). F1, the no. 1 channel optimized to detect emissions from SYBR green I.

retinochoroiditis (Contini *et al.*, 2005). Although SAG-4 DNA levels were much higher than those of MAG-1, neither gene was found after 4 months of follow up (data not shown). This suggests that the persistence of bradyzoite DNA arises from the fact that it is not rapidly cleared. The negative bradyzoite PCR amplification at 4 months indicates spontaneous conversion and cyst development, and the ending of acute disease.

Further evaluation of more clinical specimens is required. LC-PCR investigation of PBMCs with alternative gene targets to detect *T. gondii* DNA could provide valuable information on the actual state of infection, especially during the post-acute phase of the disease in the immunocompetent host.

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