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Real-Time Reverse Transcription-PCR Quantification of Cytokine mRNA Expression in Golden Syrian Hamster Infected with *Leishmania infantum* and Treated with a New Amphotericin B Formulation

S. Rama Iñiguez,[1]† M. A. Dea-Ayuela,[1]† J. A. Sanchez-Brunete,[2] J. J. Torrado,[2] J. M. Alunda,[3] and F. Bolas-Fernández[1]*

Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, Madrid, Spain[1]; Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad Complutense, Madrid, Spain[2]; and Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain[3]

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A real-time quantitative reverse transcription-PCR assay was developed for the quantification of cytokine mRNA expression in the golden Syrian hamster *Mesocricetus auratus* infected with *Leishmania infantum* and treated with amphotericin B (AMB) formulated in microspheres made of human serum albumin (HSA). Treatment was administered intravenously on days 69, 71, and 73 postinfection (p.i.) with 10^7 metacyclic promastigotes, at doses of 2 and 40 mg/kg of AMB. High infection levels were recorded for untreated animals by day 76 p.i., with parasite loads always about 2 $\log_{10}$ per gram higher in the liver than in the spleen. Treatment was highly effective with both doses, but at 40 mg/kg, almost complete parasite elimination was achieved. mRNA expression of gamma interferon (IFN-γ) and, to a lesser extent, tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) in spleen cells was up-regulated in most animals of the untreated group. The mRNA expression of interleukin-4 was strongly down-regulated in untreated as well as treated infected animals. Treatment with the lower dose of AMB-HSA down-regulated the mRNA expression of IFN-γ and TNF-α, with no effect on the deactivating cytokine TGF-β. In contrast, treatment with the higher dose (40 mg/kg) of the formulation caused moderate up-regulation of IFN-γ and TNF-α and strong suppression of TGF-β. Treatment of noninfected animals did not alter the cytokine expression pattern with regard to untreated controls. Our results suggest that treatment of *L. infantum*-infected Syrian hamsters with highly effective nontoxic doses of AMB-HSA causes deactivation of the anti-inflammatory cytokine TGF-β, which in turn results in up-regulation of the Th1 cytokines IFN-γ and TNF-α.

Leishmaniasis is a zoonotic disease caused by an obligate intracellular parasite living in mammalian macrophages which is transmitted by means of a bite of a sandfly vector. The disease is endemic to tropical and subtropical areas as well as the Southern European countries, ranging from the American forest to the Western Asian desert and from rural to urban areas (7). About 12 million people suffer from the disease, and nearly 350 million people are at risk worldwide, with 72 million of them living in developing countries (39). Over the last 10 years, regions of endemicity have become widespread due to economic breakdowns that increased individual risk factors such as malnutrition and immunosuppression and to environmental changes that favor vector exposure. With some exceptions (visceral leishmaniasis by *Leishmania donovani* in India and cutaneous leishmaniasis by *L. tropica*), humans are accidental hosts, whereas other mammals, such as rodents and canids, are reservoirs (2). Human immunodeficiency virus and *Leishmania* coinfections have emerged in intravenous drug abusers in Mediterranean countries (29).

Laboratory animal models have been developed for detailed study of the various aspects of the disease. Among them, inbred mice have been the most commonly used, but they do not properly reproduce the clinical course of the disease in humans (1, 14). In contrast, the golden Syrian hamster (*Mesocricetus auratus*) is highly susceptible to infection by *L. donovani*, *L. infantum*, and *L. chagasi*, thus developing a fatal form of the visceral disease. The clinical features of visceral infection in the golden Syrian hamster largely correlate with the human manifestation of the disease (18, 26). Nevertheless, the model has been hampered by the lack of commercial reagents for immunological studies.

In 1998, Melby et al. (23) cloned and sequenced portions of the cytokines interleukin 2 (IL-2), IL-4, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-10, IL-12 p40, and transforming growth factor beta (TGF-β) and measured their expression in hamsters infected with *Leishmania donovani* for 56 days. They found basal expression of Th2-type cytokines (IL-4), high expression of Th1-type cytokines (IL-2, IFN-γ, and TNF-α), and a lack of mRNA expression for nitric oxide synthase (22). Later, an end-point reverse transcription-PCR (RT-PCR) assay was also implemented by Mendez et al. (24) to validate the golden Syrian hamster as a model for human hookworm infections. However, these two approaches allowed only a semiquantitative analysis of mRNA expression by densitometry. Therefore, for the present study, we developed a real-time quantitative RT-PCR assay for the quantification of mRNA expression of four cytokines (IL-4, IFN-γ, TNF-α, and TGF-β) in hamsters infected with *L. infantum*.

* Corresponding author. Mailing address: Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, Plaza de Ramón y Cajal s/n, Ciudad Universitaria, 28040 Madrid, Spain. Phone: 34 91 394 18 18. Fax: 34 91 394 18 15. E-mail: francisb@farm.ucm.es.
† S.R.I. and M.A.D.-A. participated equally in this paper.

Furthermore, this technique was applied to assess cytokine expression changes following treatment of visceral infections with relatively high doses of a new formulation of amphotericin B (AMB) (6). This formulation consists of hydrophilic albumin microspheres containing AMB. The microspheres are smaller than 5 μm, easily water dispersible, easy to reproduce, and stable in heat and relative humidity (31). They are of very low toxicity when administered intravenously to Syrian golden hamsters and promote fast clearance of the drug from plasma and its accumulation in the liver and spleen (30).

MATERIALS AND METHODS

Animals. Male golden Syrian hamsters of 60 to 70 g of body weight were purchased from Harlan Ibérica S.A. and hosted in Macrolon PANLAB cages under controlled light, temperature, and humidity conditions. Animal handling was carried out according to the Council Directive 86/609/EEC of 24 November 1986 on the approximation of lows, regulations, and administrative provisions of member status regarding the protection of animals used for experimental and other scientific purposes and the Code of Practice for the Housing and Care of Animals used in Scientific Procedures 1989.

Parasite. The autochthonous isolate M/CAN/ES/96/BCN150 (Zymodeme MON-1) of *L. infantum* was kindly provided by C. Alonso and J. M. Requena (UAM, Madrid, Spain) in 1999. Since then, it has been maintained in our laboratory by periodic passages in golden Syrian hamsters.

Drugs and formulations. Micronized AMB of 93% potency was a gift from Bristol-Myers Squibb (Barcelona, Spain). Twenty percent human serum albumin (HSA) was obtained from Aventis Behring (Barcelona, Spain). AMB-HSA microspheres were prepared by spray drying as previously described (30). Briefly, AMB (50 mg) was dispersed in 4.49 ml of an aqueous solution containing sodium deoxycholate (41 mg), dibasic sodium phosphate (10 mg), and monobasic sodium phosphate (0.9 mg). The resulting dispersion was subjected to moderate stirring until it became a homogeneous suspension. A 20% serum albumin solution (4.49 ml) was added, and the final mixture was spray dried (inlet temperature, 165°C; feeding at 3.0 ml/min) using a Büchi B 191 spray drier (Flawil, Switzerland). Microspheres were collected and heated at 60°C for 1 h. Before drug injection, AMB-containing microspheres were dispersed in a 5% glucose-water solution. The formulation was administered by the intracardiac (i.c.) route to previously anesthetized (sodium pentobarbital; Sigma) animals.

Preparation of parasites for experimental infection. Amastigotes harvested from spleens of infected hamsters were cultured in NNN medium supplemented with penicillin (200 IU), gentamicin (200 μg/ml), and streptomycin (2 mg/ml) for 2 days up to their transformation into promastigotes. Thereafter, they were transferred to C-199 medium supplemented with 1% 10 mM adenine in 50 mM HEPES, 0.25% hemin in 50% triethanolamine, 0.348 g/liter bicarbonate, 25 mM HEPES, 20% heat-inactivated fetal calf serum (Cansera, Ontario, Canada), 100 μg/ml penicillin, and 100 μg/ml streptomycin at pH 7.2. Under these conditions, the maximum number of metacyclic forms, as determined by resistance to complement lysis, was achieved by day 7. After 7 days, the primary culture promastigotes were harvested by centrifugation at 2,000 × *g* for 15 min, washed with phosphate-buffered saline (PBS) at pH 7.2, and finally resuspended in PBS. Promastigotes were counted using a Neubauer hemocytometer, and the final suspension was adjusted to provide the appropriate numbers of promastigotes per inoculum. Each hamster was infected with 10^7 stationary promastigotes given by the i.c. route following anesthesia with sodium pentobarbital (Sigma).

Treatment of noninfected and *L. infantum*-infected Syrian hamsters with AMB-HSA microspheres. An experiment was designed to measure the effects of treatments with active and very highly active nontoxic formulations of AMB on the expression of several cytokines by spleen immune cells to approach further monitoring of the kinetics of the cellular immune response after effective treatment. Three groups of five animals each were infected with 10^7 promastigotes of *L. infantum* per animal. One group (group I) remained untreated, whereas the other two groups (groups II and III) were treated with doses of 2 and 40 mg/kg of AMB-HSA microspheres, respectively, on days 69, 71, and 73 postinfection (p.i.). These doses were selected from previous studies as highly and very highly effective, respectively, against an established infection by *L. infantum*, with no toxic effects on the host (30). One animal from group III died before treatment of unknown reasons, and the remainder were sacrificed on day 3 posttreatment (day 76 p.i.).

In order to assess the effects of this new formulation on cytokine mRNA expression in noninfected animals, a complementary experiment was added, with two groups (IV and V) of five animals each who were age and weight matched to the infected animals. These animals were treated in exactly the same way as the infected animals.

TABLE 1. Nucleotide sequences of primers and fluorogenic probes used in RT-PCR for amplification of cytokines transcripts

Cytokine and primer[a]	Sequence (5′–3′)	Product size (bp)
IFN-γ		
F	GGCCATCCAGAGGAGCATAG	68
R	CCATGCTGCTGTTGAAGAAGTTAG	
IL-4		
F	CCACGGAGAAAGACCTCATCTG	72
R	GGGTCACCTCATGTTGGAAATAA	
TGF-β		
F	GCGGCAGCTGTACATCGA	57
R	GGCTCGTGAATCCACTTCCA	
TNF-α		
F	AACTCCAGCCGGTGCCTAT	81
R	GTTCAGCAGGCAGAAGAGGATT	
HPRT		
F	AGATCCACTCCCATAACTGTAGATTTTAT	91
R	CATCCGCACCATTAATTTTTAAGTC	

[a] F, forward primer; R, reverse primer.

Estimation of parasite burden. The animals were euthanized (by overdose anesthesia) on day 76 p.i. The liver and spleen from each animal were removed and weighed, and afterwards, samples of each were homogenized in cold PBS-glucose-EDTA solution using a stainless steel tissue grinder. Cell debris was eliminated by passage through a glass wool column. The suspensions obtained were centrifuged at 3,500 rpm for 15 min at 4°C. Thereafter, the supernatants were discarded and the pellets resuspended in C-199 medium supplemented with 1% adenine, 0.2% hemin, sodium bicarbonate, 25 mM HEPES, antibiotics, and 20% fetal calf serum. Two hundred microliters of suspension was transferred to each of 96 microtiter wells containing NNN medium supplemented with antibiotics. Parasite burdens were estimated by a limiting dilution assay according to the methods of Hill et al. (17) and Titus et al. (34). The results were expressed as $\log_{10}$ values of the numbers of parasites per gram of tissue.

Preparation of spleen cell samples. Spleen samples from each animal were individually homogenized through a 40-μm stainless steel tissue grinder in RPMI medium supplemented with 2 mM L-glutamine, 10 mM HEPES, sodium bicarbonate (2.2 g/liter), and gentamicin (50 μg/ml). The cell suspension was treated with NH_4Cl at 0.8% to remove red blood cells, and thereafter the cells were washed three times in RPMI by centrifugation at 1,500 rpm for 5 min. After being washed, the cells were counted using a Neubauer hemocytometer, and their viability was determined by trypan blue staining. Cell suspensions were adjusted to a concentration of 20×10^6 cells/ml.

mRNA extraction and transformation into cDNA. mRNAs were extracted from 20×10^6 spleen cells using a QuickPrep Micro mRNA purification kit (Amersham Bioscience) following the instructions of the supplier, and the mRNA content was measured in a spectrophotometer at 260 nm. One hundred fifty nanograms of mRNA was then transformed into cDNA by reverse transcription using a First Strand cDNA synthesis kit (Amersham Bioscience). The cDNA samples were stored at −80°C until use.

DNA extraction. In order to obtain a DNA reference standard curve for cytokine expression, genomic DNAs were extracted from spleen cells from healthy hamsters using a G-Nome DNA kit (BIO 101 Systems) according to the manufacturer's instructions. The obtained DNA samples were stored at −20°C until use.

Conventional and quantitative real-time PCR. Conventional PCR was previously carried out in order to set up appropriate conditions for real-time RT-PCR. PCR was carried out in an MJ Research minicycler in a reaction mixture containing 12.5 μl of AmpliTaq Gold PCR master mix (Applied Biosystems), 2 or 2.5 μl of DNA or cDNA, respectively, and primers to a final concentration of 300 nM. Primer design was done based on primers published by Melby et al. (23), using Primer Express software (Applied Biosystems). The primer sequences are recorded in Table 1.

For hypoxanthine phosphoribosyltransferase (HPRT), IL-4, IFN-γ, and TNF-α, the amplification cycles were as follows: 10 min at 95°, 40 cycles of 1 min

TABLE 2. Parasite burdens in liver and spleen homogenates from hamsters 3 days after treatment with HSA-amphotericin B microspheres at doses of 2 and 40 mg/kg[a]

Treatment group	Animal no.	Log_{10} parasites/g organ[b]	
		Spleen	Liver
I (untreated)	1	5.58	7.67
	2	5.11	7.84
	3	4.76	7.9
	4	6.82	6.94
	5	6.32	7.71
Mean (SD)		5.71 (0.75)	7.61 (0.34)
II (2 mg/kg)	1	4.65	6.59
	2	4.76	6.04
	3	4.46	6.66
	4	3.55	6.04
	5	4.90	6.66
Mean (SD)		4.46* (0.47)	6.39* (0.29)
III (40 mg/kg)	1	2.04	4.59
	2	2.39	3.23
	3	ND	ND
	4	3.25	5.93
	5	2.86	5.73
Mean (SD)		2.63* (0.45)	4.87* (1.07)

[a] Treatment was given by the intracardiac route on days 69, 71, and 73 after infection with 10^7 promastigotes of *L. infantum*. Estimations of parasite burdens were done by the limiting dilution assay.

[b] *, significantly different from untreated group ($P < 0.05$). ND, not determined.

at 95°C, 1 min at 56°C, and 1 min at 70°C, and, lastly, 7 min at 72°C. The same amplification conditions were used for TGF-β except that the annealing temperature was 60°C instead of 56°C. The amplification products were separated by electrophoresis in 3% agarose gels at 80 V and visualized by staining with ethidium bromide.

Real-time quantitative PCR was carried out with 25 μl of SYBR green PCR master mix (Applied Biosystems), 10 μl of DNA or 2.5 μl of cDNA, and primers at a final concentration of 300 nM in a final volume of 50 μl, using the same amplification conditions as those described for conventional PCR. cDNAs from noninfected and nontreated hamsters were use as "comparator samples" for quantification of those corresponding to test samples. All quantifications were normalized to the housekeeping gene HPRT. A no-template control with no genetic material was included to eliminate contaminations or nonspecific reactions.

Data analysis. Sample analysis was carried out with the ABI Prism 7700 sequence detection system, using 96-well optical reaction plates with optical caps (Applied Biosystems). Standard curves were generated from comparator samples made from decreasing amounts of genomic DNA (10-fold dilutions) to monitor the efficiency of real-time RT-PCR for each assay. Prior to acceptance of data for quantitative work, at least four of the five standard curve dilutions in an assay were required to yield a specific product (based on dissociation curve analysis), and no product could be seen in the no-template control. The quality of the standard curves can be judged from their slopes and correlation coefficients (see Results).

The cycle threshold (C_T) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise). Differences in gene expression were calculated by the comparative C_T method (21). This method compares test samples to a comparator sample and uses results obtained with a uniformly expressed control gene (HPRT) to correct for differences in the amounts of RNA present in the two samples being compared to generate a ΔC_T value. Results are expressed as the degrees of difference between ΔC_T values of test and comparator samples. A difference of threefold or more is considered significant (4).

Statistical analysis. Parasitological data for treated and untreated animals were compared by the Mann-Whitney U test. Significance was given to those groups that differed with P values of <0.05. Individual immunological data from each group were compared with those from the other groups by applying the Kruskal-Wallis test (SPSS 12.0 for Windows). This test is a nonparametric analysis which ranks the values of two or more sample groups from lowest to highest and then compares the distribution of these ranks to determine if one or more of the groups are significantly different from the others.

Nucleotide sequence accession numbers. The GenBank accession numbers for the primers designed for this study are as follows: HPRT primers, AF047041; IL-4 primers, AF046213; IFN-γ primers, AF034482; TGF-β primers, AF046214; and TNF-α primers, AF046215.

RESULTS

Quality assessment of PCR and real-time PCR for cytokine mRNA expression. For quality assessment of PCR techniques, mRNA and genomic DNA samples were extracted from spleen cells taken from healthy hamsters. Figure 1 shows the amplification products of cDNAs and genomic DNA for the housekeeping (HPRT) and cytokine genes separated in agarose gels and stained with ethidium bromide. Single amplification products corresponding to the HPRT control gene and to the TGF-β, TNF-α, IFN-γ, and IL-4 genes were visualized at 91, 57, 81, 68, and 72 bp, respectively.

For real-time quantitative PCR with genomic DNA, standard and dissociation curves were constructed for housekeeping and cytokine genes. A single dissociation peak for the product of each primer pair was obtained, thus showing that the real-time RT-PCR assay was gene specific and that the results were not confounded by nonspecific amplification or primer dimers.

The slopes of standard calibration plots of the C_T values versus log genomic DNA concentrations for serial dilutions were −3.16 for IFN-γ, −3.10 for IL-4, −2.97 for TNF-α, and −3.12 for TGF-β. These were quite close to the slope obtained with the control gene HPRT (−3.30). The mean correlations (r^2) of detection for all transcripts were 0.986 for IFN-γ, 0.996 for IL-4, 0.999 for TNF-α, 0.999 for TGF-β, and 0.995 for HPRT.

Assessment of effectiveness of treatment with AMB-HSA microspheres. The average parasite load in untreated and treated animals was always about 2 log_{10} per gram higher in the liver than in the spleen. High effectiveness was recorded with both doses of AMB-HSA ($P < 0.05$), but with 40 mg/kg, parasitological cure was almost achieved (99.96 and 99.29% reduction in loads in the spleen and liver, respectively, compared

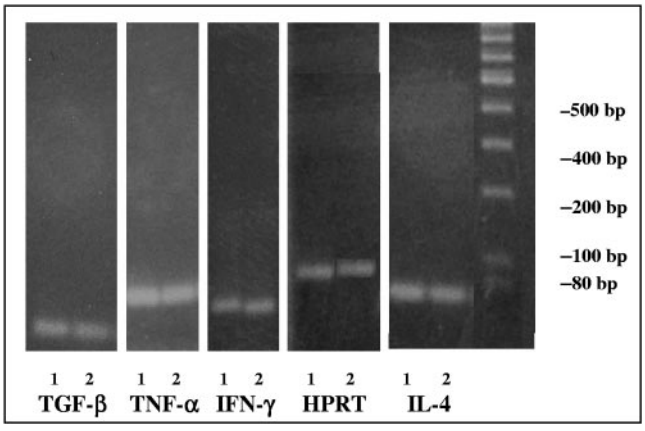

FIG. 1. Amplification products of cDNA (1) and DNA (2) control samples were separated in agarose gels and stained with ethidium bromide. Single amplification products corresponding to the HPRT control gene and the TGF-β, TNF-α, IFN-γ, and IL-4 genes were visualized at 91, 57, 81, 68, and 72 bp, respectively.

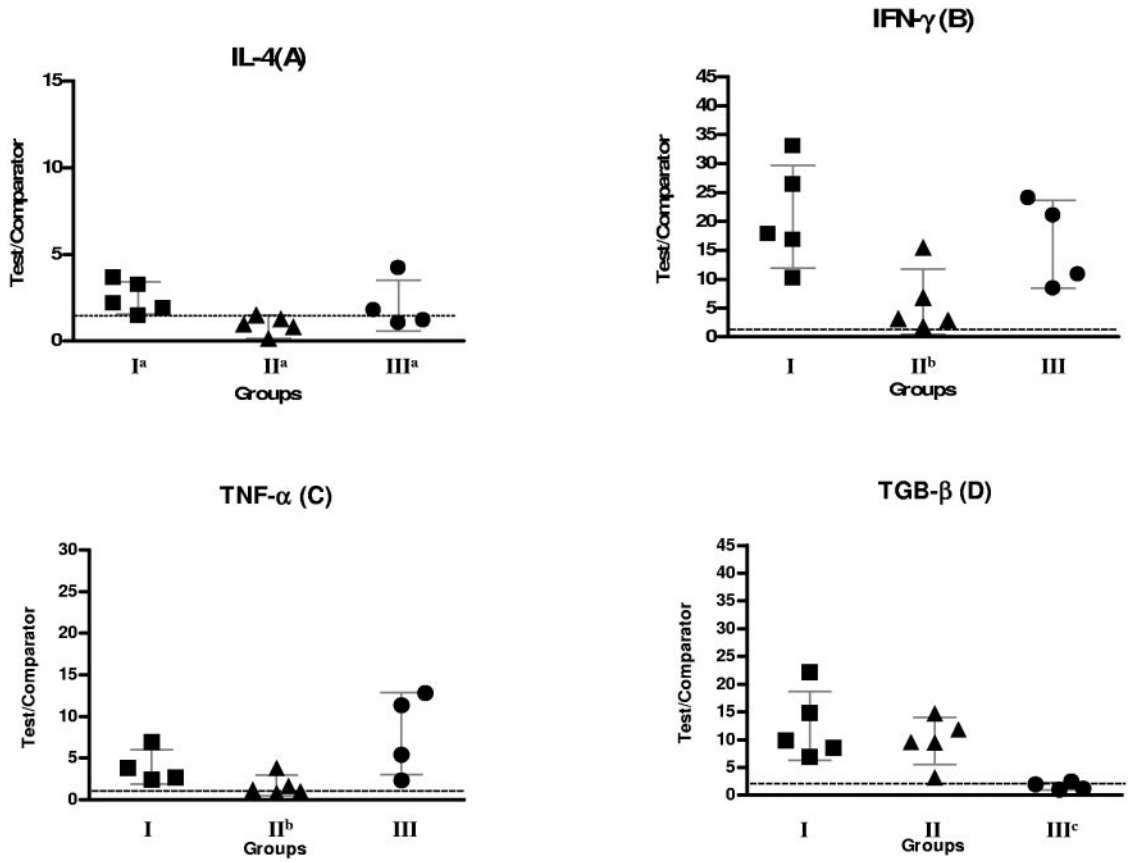

FIG. 2. Relative mRNA expression levels of IL-4 (A), IFN-γ (B), TNF-α (C), and TGF-β (D) in spleen lymphocytes from Syrian hamsters infected by *L. infantum* (group I) or infected and treated with AMB-HSA at 2 mg/kg (group II) or 40 mg/kg (group III). Treatment was given on days 69, 71, and 73 after infection, and spleen cells were collected following sacrifice on day 3 after treatment. Results are expressed as differences between ΔC_T values of test and comparator samples. The horizontal dotted lines represent the ΔC_T of the comparator samples, to which the value of 1 was arbitrarily assigned. Truncated vertical lines represent the standard deviation ranges for mean values. a, mean values for groups I, II, and III are not significantly different from each other ($P > 0.05$); b, mean values for group II are significantly different from those for groups I and III ($P < 0.05$); c, mean values for group III are significantly different from those for groups I and II ($P < 0.05$).

to the untreated group). One and 3 $\log_{10}$ per gram fewer parasites were recorded for animals treated with 2 and 40 mg/kg, respectively, than for untreated animals (Table 2). In parallel experiments, it was shown that when treated with AMB microspheres at 2 mg/kg, all animals eventually developed clinical symptoms of the disease in a similar way to those left untreated, whereas with 40 mg/kg, all animals remained asymptomatic and parasitologically negative (data not shown).

Comparison of mRNA cytokine expression by spleen cells in infected and noninfected hamsters treated with AMB-HSA microspheres. Very low levels of IL-4 mRNA expression were observed in animals of group I by the day of observation (76 days p.i.), with values slightly surpassing the positive level (test/comparator ratio of >3) in two of five animals. Treatment did not significantly change the mRNA expression pattern for this cytokine ($P > 0.05$) (Fig. 2A).

mRNA expression for IFN-γ was highly up-regulated in all group I animals by day 76 postinfection (Fig. 2B). A significant decrease ($P < 0.05$) was observed in most animals treated with 2 mg/kg of AMB-HSA microspheres (group II), with test/comparator ratios dropping under the positive threshold level for three of five animals. In contrast, in animals treated with the high dose of the new formulation, IFN-γ mRNA expression was clearly up-regulated ($P < 0.05$), with test/comparator ratios rising to values near those recorded for untreated animals. A correlation between IFN-γ mRNA expression test/comparator ratios and parasite loads was apparent only in some cases, mainly for the liver ($P < 0.05$), although with lower test/comparator ratio values, the pattern of mRNA expression for TNF-α was quite similar to that for IFN-γ (Fig. 2C).

TGF-β mRNA expression was clearly up-regulated in all animals of groups I and II, although with great variation in the former. In contrast, mRNA expression for this cytokine was drastically suppressed in all animals of group III ($P < 0.05$) (Fig. 2D).

Treatment without infection did not affect the pattern of mRNA expression in any animals of groups IV and V (treated with 2 and 40 mg/kg, respectively), as test/comparator ratios always remained below 3 (Fig. 3A to D).

DISCUSSION

In the present study, we have successfully adapted a two-step SYBR green I real-time RT-PCR assay to measure cytokine mRNA expression in the golden Syrian hamster to assess im-

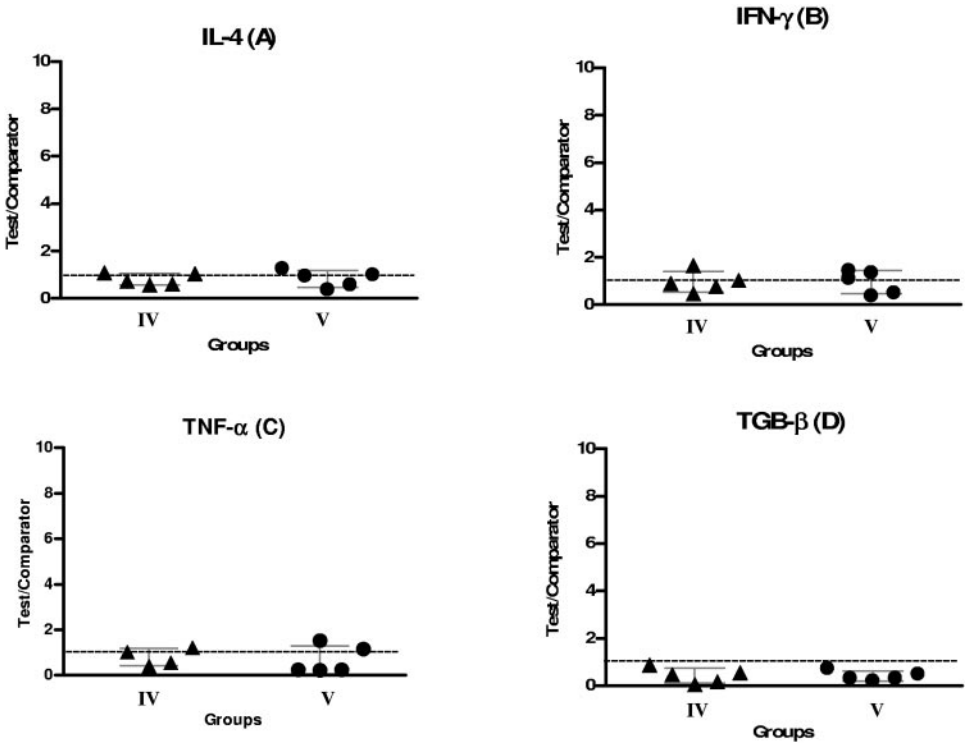

FIG. 3. Relative mRNA expression levels of IL-4 (A), IFN-γ (B), TNF-α (C), and TGF-β (D) in spleen lymphocytes from noninfected Syrian hamsters treated with AMB-HSA at 2 mg/kg (group IV) or 40 mg/kg (group V). Treatment was given on days corresponding to days 69, 71, and 73 after infection (for infected groups), and spleen cells were collected following sacrifice on day 3 after treatment. Results are expressed as differences between ΔC_T values of test and comparator samples. The horizontal dotted lines represent the ΔC_T of the comparator samples, to which the value of 1 was arbitrarily assigned. Truncated vertical lines represent the standard deviation ranges for median values. Treatment did not affect the mRNA cytokine expression patterns of both groups compared to healthy untreated animals (test/comparator ratio increase/decrease of <3).

munological changes following treatment of an established visceral infection by *L. infantum* with a new formulation of AMB. This relatively new technique offers several advantages over Northern blotting or conventional RT-PCR, since it requires less RNA and provides quantitative results. It also has a similar sensitivity and reproducibility to fluorescence methods (e.g., TaqMan probes) but is less expensive than probe-based detection methods, which require a new probe for each amplicon under investigation (4). Real-time RT-PCR can be performed in one step or two steps, in which cDNA synthesis and PCR amplification occur in separate tubes. The two-step real-time RT-PCR method has been reported to be more sensitive than the one-step method and is less prone to the production of primer-dimer artifacts and contamination with genomic DNA (4, 36). The quality of RT-PCR data was assessed by measuring the efficiency of PCR and the correlation coefficients of standard curves. The specificity of SYBR green detection of PCR products was verified by melting-curve analysis and agarose gel electrophoresis.

Our results provide evidence that real-time RT-PCR with SYBR green I is suitable for the quantification of cytokine gene transcription in hamsters, making this animal model useful for immunological testing, not only for *Leishmania* and other parasitic infections but also for viral, fungal, or bacterial infections.

Melby et al. (22, 23) measured cytokine expression in splenic lymphocytes from hamsters infected with 5×10^6 purified amastigotes of *L. donovani* on days 7, 14, 28, and 56 p.i. They demonstrated that the progression of the infections occurred in the presence of mRNA expression of Th1 cytokines (IFN-γ and IL-12) from the first week. The IFN-γ response was similar to that found in humans (19) and was in concordance with previous studies by Gifawesen and Farrell (11), who demonstrated the transference of delayed-type hypersensitivity by T cells from infected to healthy animals, thus suggesting that the IFN-γ expression associated with the control of infection in the murine *L. major* (16) and *L. donovani* (32) models would not be sufficient to control the replication of the parasite in the golden hamster. In our work, IFN-γ mRNA expression was also highly up-regulated by day 76 p.i. in all animals of the control group, in spite of their high level of infection. Therefore, the failure of IFN-γ to control multiplication of the parasite in the hamster could be due to an insufficient effector cell response to this cytokine, as suggested by Melby et al. (23). Three days after treatment at the low dose of AMB-HSA (2 mg/kg), IFN-γ mRNA expression was strongly down-regulated in four of five animals, suggesting that, at least in early steps, an active infection is needed to trigger IFN-γ production. However, the highly effective treatment achieved with 40 mg/kg caused a marked increase in IFN-γ mRNA expression in all animals exhibiting test/comparator ratios similar to those of the infected control group. The apparent dose-dependent effect of this new formulation on spleen cell activation is discussed below.

TNF-α is a proinflammatory cytokine that can have both

protective and pathological roles. TNF-α acts synergistically with IFN-γ to activate macrophages to kill *Leishmania* (33). Melby et al. (23) found that mRNA for TNF-α increased within 1 week of infection but that, later, the level of expression of this cytokine decreased. In our study, mRNA expression for TNF was also lower than that for IFN-γ in all groups, but the pattern of expression, particularly in infected and treated animals, was highly coincident. These observations may indicate a common effect of treatment on both gene expression levels. A positive effect of AMB on TNF-α gene expression has been supported by various studies performed either in vivo or in vitro (5, 35, 38), with this effect being associated with side effects (e.g., fever) observed during treatment with amphotericin B. In our work, these proinflammatory effects of the drug per se were not observed, as changes in mRNA expression were not evident in noninfected hamsters. It was reported that the immunomodulating activity of AMB can be modified by changes in its molecular aggregation stage induced by heat (15) or by the use of conjugates as carriers. Thus, Larabi et al. (20) showed that the incorporation of AMB into lipid carriers (e.g., liposomes or lipid complexes) reduced NO and TNF-α production. Falk et al. (10) reported that in various mouse organs, the proinflammatory and apoptotic actions of amphotericin B were neutralized by arabinogalactan conjugates. However, the same conjugate increased the production of TNF-α in vitro by mouse peritoneal macrophages and human monocytes, with no effect on IFN-γ and NO release (9). Therefore, it seems likely that the immunostimulating properties of AMB depend on the formulation as well as the way in which it is presented to macrophages. Likewise, in our in vivo study, it may be that the proinflammatory activity of amphotericin B on splenic macrophages was blocked by encapsulation in HSA microspheres.

Melby et al. (23) did not see a significant increase in IL-4 mRNA expression through the course of infection with regard to uninfected controls, and thus the strong basal expression of this Th2 cytokine in healthy hamsters contrasts with what has been found in mice and rats. In our study, IL-4 mRNA expression was down-regulated in infected, untreated animals, with negative values for three of five hamsters. In addition, effective treatment did not significantly change the IL-4 expression pattern. In other models, such as with *Ancylostoma ceylanicum*, the activation of IL-4 during the patent period was also very weak in comparison to that of IL-10 (24). As pointed out by Melby et al. (23), the behavior of IL-4 gene expression suggests that the regulatory sequence in the hamster gene may be different from those of other species.

IL-10 and TGF-β are potent inhibitors of macrophage activation and killing of *Leishmania* organisms (8, 3, 37). Using golden hamsters, Melby et al. (22, 23) found increasing levels of expression of mRNA for IL-10 as the infection progressed, suggesting that IL-10 may play a role in the progressive disease seen in the hamster. Concerning TGF-β, they found quite high basal levels, with a slight increase throughout infection. These relatively high levels of constitutive cytokine in the liver and spleen could inhibit the expression of nitric oxide synthase by macrophages (probably in an autocrine manner) and therefore promote susceptibility to infection (22, 23). Later, the importance of TGF-β in the suppression of cellular immunity and the subsequent increase in susceptibility to infection in murine and hamster visceral leishmaniasis was reinforced (12, 25, 28, 40). In our study, TGF-β mRNA expression was up-regulated in all animals of the untreated group and the group treated with the lower dose (2 mg/kg) of the formulation. In contrast, in the group treated with the higher dose, TGF-β mRNA expression was strongly suppressed, with no positive values recorded for any of the five animals. This observation inversely correlates with the expression patterns of IFN-γ and TNF-α observed for this group, thus reinforcing the antagonistic roles of these cytokines.

Globally, our results on cytokine expression in infected hamsters are in agreement with those previously reported by Melby et al. (22, 23). We can hypothesize that the ability of the golden hamster to produce active TGF-β could be taken by *Leishmania infantum* as an advantage to neutralize the action of Th1 cytokines (IFN-γ and TNF-α) on parasite killing by macrophages via NO triggering. Partial elimination of the infection with a noncuring dose of AMB-HSA microspheres down-regulates Th1 cytokines without affecting TGF-β expression, and therefore animals progress to eventual disease, with death by month 4 posttreatment (unpublished data). In contrast, treatment with the highly effective dose of the formulation down-regulates TGF-β, which in turn up-regulates IFN-γ and TNF-α. This dual effect would create a clinical cure in most animals (unpublished data). The reason for this dual effect could be a dose-dependent immunomodulatory effect of the new formulation similar to that observed by Reyes et al. (27) with liposomal AMB on human peripheral blood mononuclear cells. Liposomal AMB maintained increased nonspecific proliferative responses against fungal infections at doses that are toxic for the conventional nonliposomal formulation (deoxycholate AMB). However, our results for noninfected animals clearly show that without the concurrence of the parasite or its products, AMB-HSA, regardless of the dose, does not affect cytokine expression by hamster splenocytes. Therefore, this immunomodulating effect seems to be more parasite than drug/formulation dependent.

In summary, using a Syrian hamster model system of visceral infection with *L. infantum*, we demonstrated that effective eradication of an established infection is achieved by using high doses of AMB formulated in HSA microspheres without causing toxic damage to the host. As a result, the supposed massive killing of the parasite achieved with this high-dose therapy may cause deactivation of anti-inflammatory cytokines such as TGF-β (probably through strong deprivation of some active excreted/secreted parasite antigens). This in turn results in the corresponding increase of the proinflammatory IFN-γ and TNF-α factors, which eventually help in resolution of the infection. In line with this hypothesis, up-regulation of IL-10, the other Th1 deactivating cytokine, occurred in mesenteric lymph nodes of hamsters during patency of an *Ancylostoma ceylanicum* infection (24). An inhibitor of metalloproteases is abundantly secreted by adult stages of *Ancylostoma caninum* (41). Since mammalian tissue inhibitors of metalloproteases were shown to stimulate IL-10 production by Guedez et al. (13), the possibility remains that adult hookworms directly stimulate host IL-10 production (24).

To approach a confirmation of our speculative hypothesis, proteomic studies are under way for the identification of factors released by *Leishmania* amastigotes following in vitro treatment with AMB-HSA.

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