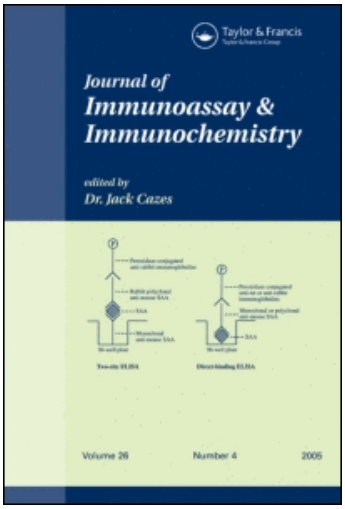

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B. Ferrua[a]; Y. Le Fichoux[a]; I. Suffia[a]; D. Rousseau[a]; C. Roptin[a]; J. Kubar[a]
[a] Laboratoire de Parasitologie, IFR 50, Chemin de Vallombrose, Nice Cedex 02, France

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QUANTITATION OF *LEISHMANIA INFANTUM* IN TISSUES OF INFECTED BALB/c MOUSE BY SANDWICH ELISA

B. Ferrua,[1,*] Y. Le Fichoux,[1] I. Suffia,[1] D. Rousseau,[1] C. Roptin,[2] and J. Kubar[1]

Groupe de Recherche en Immunopathologie
de la Leishmaniose (EA 2675),
[1]Laboratoire de Parasitologie, IFR 50,
[2]Laboratoire de Pharmacologie, Faculté de Médecine,
Chemin de Vallombrose, 06107 Nice Cedex 02, France

ABSTRACT

In this report, a sandwhich ELISA was developed to quantify spleen and liver burdens from *L. infantum*-infected BALB/c mice. Amastigote antigens obtained following Nonidet P40 extraction of parasite-harbouring tissues were captured by anti-*L. infantum* human IgG insolubilized onto microtiter plate and subsequently revealed with anti-*L. infantum* F(ab)' fragments labelled with peroxidase. The method was easy to perform, precise and capable to specifically and accurately detect 5 x 10^4 amastigotes/100 mg tissue.

Parasite burdens from infected BALB/c mice, in various conditions, were measured by ELISA and Giemsa-stained touch imprint reference methods, and compared. Both techniques agreed well with close values for liver burdens, but the spleen loads measured by the ELISA were, on average, 10.7

* Corresponding author.

times higher than those calculated from imprints. This difference was attributed partly to the underestimation brought by Stauber's formula. However, it did not preclude the usefulness of this newly developed test, since results obtained in kinetics studies and evaluation of the efficiency of leishmanicidal drugs allowed us to draw identical conclusions.

INTRODUCTION

Leishmania are obligate intracellular protozoan parasites of macrophages which cause a spectrum of human diseases,(1) including localized self-healing or diffuse skin lesions, mucosal manifestations or severe visceral diseases, the latter being fatal if untreated. Visceral leishmaniasis due to *Leishmania infantum* is endemic in the Mediterranean basin and is considered as an opportunistic infection in HIV-infected subjects.(2,3). Clinical symptoms include fever, paleness, and splenomegaly. In addition to the detection of specific antibodies by ELISA, immunofluorescence, direct agglutination or western blotting, the definitive diagnosis of leishmaniasis currently depends on the identification of characteristic amastigotes in tissue or isolation of promastigotes by culture.

The identification and/or enumeration of parasites in tissues is crucial in experimental models(4) for monitoring the time-course distribution of the parasites in target organs, and evaluating the efficacy of a new chemotherapy or protective effect of potential vaccines. For this purpose, in most experimental studies on visceral leishmaniasis, the average number of amastigotes *per* nucleated cell has been determined by microscopic examination of Giemsa-stained smears or touch imprints, and the parasitic load, extrapolated using Stauber's formula(5) previously established for liver tissue, and which takes into account the number of cells per mg tissue.

Recently, (6,7) quantification of *Leishmania* load has been performed by sensitive limit dilution culture, based on the *in vitro* transformation of amastigote form into extracellular promastigote form, and theoretically capable of detecting as little as one parasite/mg tissue.(8) By another approach, *L. infantum* could be evidenced by polymerase chain reaction (PCR) in blood of asymptomatic carrier humans(9) and dogs(10) or HIV-positive patients,(11) although, to our knowledge, no quantitative PCR has, until now, been reported for monitoring *L. infantum* infection. However, these techniques are rather tedious and cumbersome to perform, presenting some drawbacks such as the necessity of a long incubation period for limit dilution culture technique or the need of expertise for examining touch

imprints and, therefore, are not well adapted to large series occurring in experimental models.

Herein, we present an original and convenient method for quantifying *L. infantum* in tissues of experimentally infected BALB/c mice. The technique is based on the measurement of solubilized *L. infantum* antigens, obtained following detergent-treatment of amastigote-harbouring tissues, by colorimetric sandwich ELISA, set up with a high titer human anti-*L. infantum* immune serum.

EXPERIMENTAL

Materials

Nonidet P40 (NP40), Triton X-100 detergent, Phenylmethylsulfonyl fluoride (PMSF), Aprotinin protease inhibitors, maleimide-activated peroxidase (ref P 1709), hydrogen peroxide, o-phenylenediamine dihydrochloride (OPD) and Schneider's insect medium were obtained from Sigma (Sigma France, L'Isle-d'Abeau Chesnes, France). Percoll cell separation medium and Protein A-Sepharose were purchased from Pharmacia (Pharmacia Biotech France, Saclay, France). Bovine serum albumin (BSA) fraction V was from Boehringer (Boehringer France, Meylan, France).

Parasites, Infection, and Tissue Samples

The livers and spleens from *L. infantum*-infected BALB/c mice used in this report originated from various studies already published by our group.(12,13,14) In all cases, amastigote and promastigote forms of the parasite were prepared and the mice infected as follows: *L. infantum* MON1 (MHMO/FR/94/LPN101) were routinely maintained in Syrian hamsters by serial inoculations of hepatic amastigotes, and purified from spleen by gradient density centrifugation on Percoll. The promastigote form was routinely grown in complete Schneider's medium as described(7) and used for infection after two *in vitro* passages, as stationary-phase cells from 7-day-old cultures (2.5×10^7/ml). Prior to the infection, the metacyclic promastigotes were washed three times with saline and suspended at 2×10^8 cells/mL.

Seven-week-old female BALB/c mice (Iffa Credo, Arbresle, France) were infected by inoculation of 10^8 parasites into the caudal vein and maintained in a positive pressure chamber (Esi-Flufrance, Wissous,

France) for the duration of various experiments. Non infected littermates, used as age-matched controls, were bred under the same conditions.

At the end of the experiments, mice were anaesthetized with sodium thiopental and sacrificed. Livers and spleens were recovered under sterile conditions and *Leishmania* infection was measured by blinded microscopic enumeration of Giemsa-stained liver and spleen touch prints. The parasite burdens were expressed in Leishman Donovan Units (LDU) calculated from Stauber's formula (Stauber): LDU = average number of amastigotes/nucleated cell x organ weight (mg) x 2 x 10^5. A piece of each organ (50-100 mg) was saved and immediately processed, as described below, for ELISA measurement.

Preparation of ELISA Reagents

Sera from patients with patent leishmaniasis and showing by Western-blot analysis typical multiple-band patterns against *L. infantum* antigens crude preparation(15) were tested by classical ELISA as already described.(13) A particular serum sample (Nam), showing a high titer antibody, was selected for the whole study. From 20 mL serum, 320 mg of IgG fraction was prepared by Protein A-Sepharose affinity chromatography and precipitation with ammonium sulphate. This fraction was stored at 25 mg/mL in 50% glycerol at $-20^{\circ}C$ and selected for coating, or was further digested with pepsin(16) and filtered on ACA 44 exclusion chromatography column in order to prepare $F(ab)'_2$ fragments.

Five mg of $F(ab)'_2$ fragments in 0.1 M phosphate buffer, pH 6.0, were then reduced with 2-mercaptoethylamine and conjugated to maleimide-activated peroxidase following the method of Ishikawa et al.(16) and according to the manufacturer's instructions (Sigma). Unreacted material was removed by ACA 44 gel filtration chromatography and the peroxidase-labelled anti-*L. infantum* conjugate was supplemented with 10 mg/mL BSA and 0.01% thimerosal, filtered on a 0.22 μm membrane and stored at $-20^{\circ}C$ in 50% glycerol. Under this form, this reagent was stable throughout the whole study.

Anti-*L. infantum* IgG-Coated Microtiter Plates

Anti-*L. infantum* human IgG (Nam) was diluted to 25 μg/mL with sterile 100 mM phosphate buffer, pH 7.2, (PB) and 0.15 mL of the coating solution was delivered into 96-well microtiter plates (High binding Greiner ELISA plates, Dutscher, Brumath, France). The plates were covered with a

plastic sealer and incubated for 3 hrs at 25° C, washed 5 times with PBS 0.1% Tween 20 (PBS-Tween), and saturated with the assay buffer, described below, for 30 min at room temperature. Coated plates were briefly washed with PBS-Tween and used immediately or stored for a maximum of one week at +4°C filled with this last buffer.

L. infantum Standard and Detergent Treatment of Tissue Samples

Purified promastigotes and amastigotes, prepared as described above, were enumerated with a Malassez haemocytometer, solubilized at 10^8/ml in PBS containing 1% NP40, 2 mM EDTA, 2 mM PMSF, and 1000 U/mL aprotinin (solubilization buffer), and assayed for protein content against BSA standard (MicroBCA, Pierce, Rockford, IL, USA). 10^6 Amastigotes and promastigotes were found to contain 2 μg and 5 μg of BSA protein equivalents, respectively. These preparations were stored in 50% glycerol at −20° C at 160 μg/mL concentration and were referred to as *L. infantum* amastigote or promastigote standard stock solutions.

BALB/c liver and spleen tissue samples to be analyzed were thoroughly homogenized at 100 mg/mL in solubilization buffer, using a 3 mL tapered tissue grinder (Wheaton, Millville, NJ, USA) and the suspension was centrifuged at 15,000 g for 20 min. The unsolubilized material was discarded and the supernatant was serially diluted with PB containing 1% skimmed dry milk, 0.12% Triton X-100, 0.02% thimerosal, 0.2% v/v chloroform and 50 μg/mL phenol red (assay buffer). *L. infantum* amastigote standards, ranging from 0 to 12 μg/mL, were prepared by serial dilutions of the stock solution in assay buffer.

ELISA Protocol

Duplicate 0.1 mL aliquots of amastigote standards in assay buffer, or serially diluted NP40 liver and spleen extracts, were dispensed into the anti-*L. infantum* IgG-coated wells. The plate was sealed and incubated overnight at room temperature. After washings with PBS-Tween, 0.1 mL of peroxidase-labelled anti-*L. infantum* F(ab)', diluted in assay buffer (ca 500 ng) were dispensed, and the plate incubated for 1h 30 min at room temperature.

After washing as above, bound enzymatic activity was revealed with 0.08 mL of chromogenic substrate solution (0.1 M phosphate citrate buffer, pH 5.5, H_2O_2 0.02%, OPD 2HCl 3 mg/mL), for 30 min at room temperature. The reaction was quenched with 0.1 mL of 2 N H_2SO_4 and the absorbances read at 492 nm in an automatic plate reader, with a reference filter at

630 nm. Absorbances were plotted versus *L. infantum* amastigote standard concentrations.

RESULTS

Standard Curves and Assay Precision

As the ELISA is likely to measure soluble material, *L. infantum* standard was expressed rather in μg/mL than in parasite/mL, and results obtained with tissue samples were expressed as μg of amastigote protein equivalents per mL, corresponding to 100 mg of NP40-extracted tissue. For the correlation studies, the number of parasites per organ was then extrapolated, taking into account the organ weight and the correspondence of 2 μg protein $= 10^6$ amastigotes as established above in the methods section.

Fig. 1 shows a typical standard curve for the *L. infantum* ELISA. The curve is linear over the studied range, with a signal/background ratio higher than 100 and the threshold sensitivity (calculated as the smallest concentration of amastigote proteins giving an absorbance statistically different, with a 99% confidence, from that of zero standard) was 0.05 μg/mL, corresponding to 2.5 x 10^4 amastigote *per* 100 mg tissue. In practice, 5 x 10^4 amastigotes/100 mg tissue could be reproducibly detected by ELISA.

A standard curve, derived with promastigote standards, was superimposable to that obtained with amastigote (data not shown). Assay precision was estimated by measuring three samples of NP40-extracted spleen or liver spanning the useful range, 10 times each, in the same series, or on five different occasions. Coefficients of variation ranged from 3.1% to 6.4%.

Assay Specificity

NP40-tissue extracts from spleen and liver of uninfected age-matched controls didn't contain assayable material (Fig.1), thus indicating that, under our assay conditions, no cross-reacting antigens were detectable in these target organs. Standard curves derived with either NP40-solubilized isolated amastigotes or NP40-solubilized liver or spleen from infected animals were linear (Fig.1) and, therefore, superimposable, strongly suggesting that the human antibody selected for the ELISA recognized amastigote proteins in the standard, and those present in parasite-harbouring tissues, with a similar affinity.

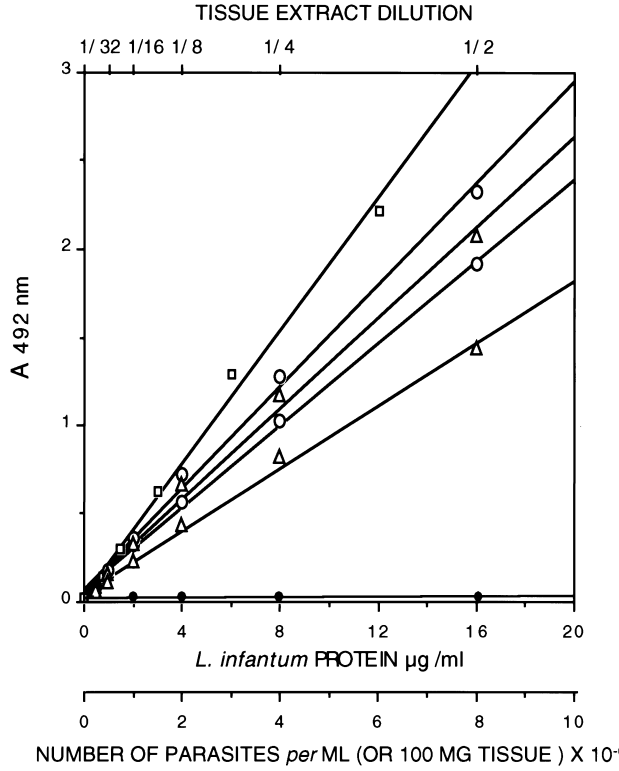

Figure 1. Representative standard curves for the *L. infantum* ELISA. Standard curve derived with NP40-solubilized amastigote standard (open squares) and curves built with dilutions of NP40 extracts obtained from spleen and liver of uninfected BALB/c mouse (filled circles) and from 2 spleens (triangles) or 2 livers (open circles) of *L. infantum*-infected BALB/c mouse. Amastigote standards are expressed as µg/mL of *L. infantum* proteins or as the corresponding number of amastigotes *per* mL, equivalent to 100 mg tissue.

Recovery experiments were performed by spiking NP40-solubilized uninfected spleen or liver with known and variable concentrations of amastigote standards. Percentages of recovery (Table 1) were in the range of 72% to 78% for undiluted samples, regardless of the tested concentration of amastigote standard, and reached more than 90% once the samples were half-diluted with the assay buffer, thus indicating the lack of a strong matrix effect and/or amastigote antigens-binding material in these NP40-extracted organs.

Table 1. Recovery Experiments: NP 40 Extracts from Uninfected Spleen* or Liver* Were Spiked with Amastigote Standard at the Indicated Concentrations and Assayed by ELISA, Undiluted or Diluted 1:2 with the Assay Buffer.

	Spleen		Liver	
Added (mg/mL)	0.8	8.0	0.8	8.0
Recovered (mg/ml) undiluted sample	0.58 (72.5)	6.30 (78.7)	0.62 (77.5)	5.70 (71.2)
Sample diluted 1:2	0.73 (91.2)	7.30 (91.3)	0.70 (87.5)	7.10 (88.7)

* Number in brackets indicate the percentage of recovery.

Correlation Studies

Spleen and liver parasite burdens of various infected BALB/c mice, originating from different experiments quoted in the Experimental section, were measured both by ELISA and touch imprints and compared. A good agreement (Fig. 2) was found ($r^2 = 0.889$) between both techniques in the case of measurement of liver parasite burdens. In addition, the slope of the regression curve was close to 1, thus validating the ELISA approach.

In contrast, in the case of spleen burden assessment, although both techniques still agreed well, ($r^2 = 0.843$) the ELISA apparently gave higher parasite burdens than those extrapolated from parasite counts using the unmodified Stauber's formula, with an average factor (calculated as the ratio: slope of the regression curve (liver)/slope of the regression curve (spleen)) of 10.7. However, this difference was not detrimental to the usefulness of the developed ELISA. Indeed, Fig. 3 shows that similar patterns of time-course distribution of *L. infantum* in liver and spleen of infected BALB/c mice were evidenced by both techniques. Furthermore, the ELISA appeared to be as sensitive as touch imprint (Fig 4) in detecting the parasite burden decrease due to the long-term efficacy of miltefosine and, in particular, in evidencing its protective effect in spleen as compared to glucantime.(14) Again, close values were noticed with both methods for livers whereas, for spleens, ELISA values exceeded, by approximately one log, those of the touch imprints.

Finally, to prove the general suitability of high-titer human sera from VL patients for developing sensitive and accurate immunoassays, a second

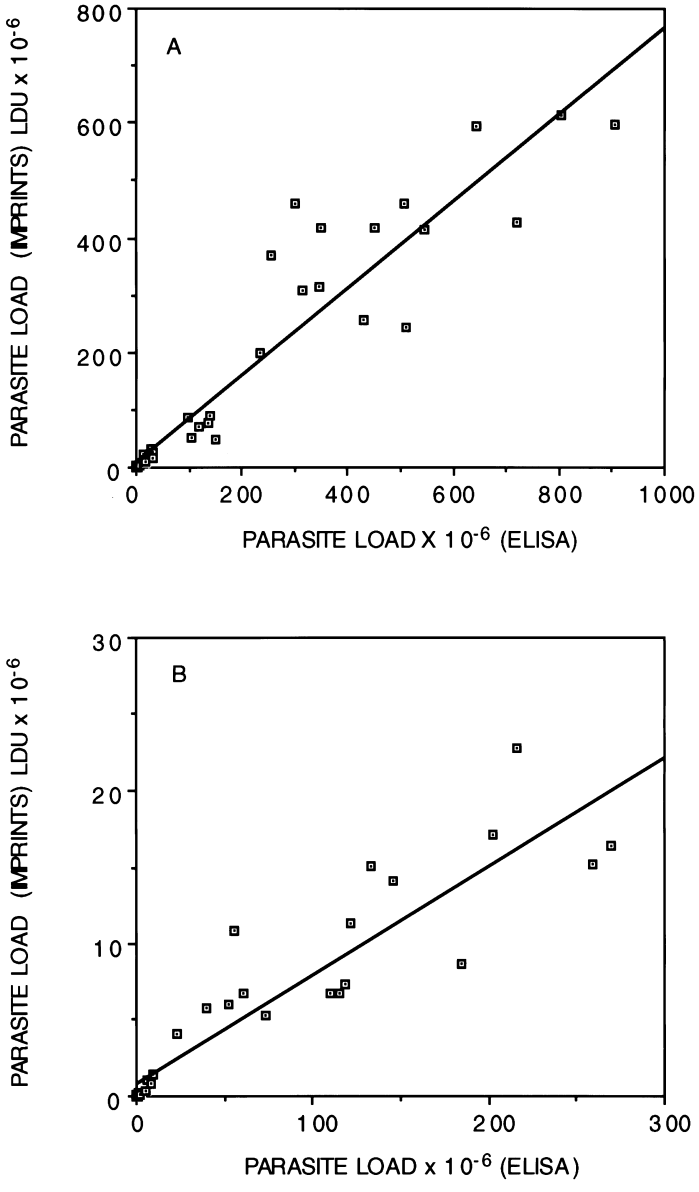

Figure 2. Comparison of ELISA and touch imprint. 43 liver (2A) and 33 spleen (2B) samples from *L. infantum*-infected BALB/c mice were analyzed by ELISA and touch imprints. Coefficients of correlation and equations of the regression lines were $r^2 = 0.889$; $y = 0.75x + 10.08$ and $r^2 = 0.843$; $y = 0.070x + 0.83$ for measurements in liver and spleen, respectively.

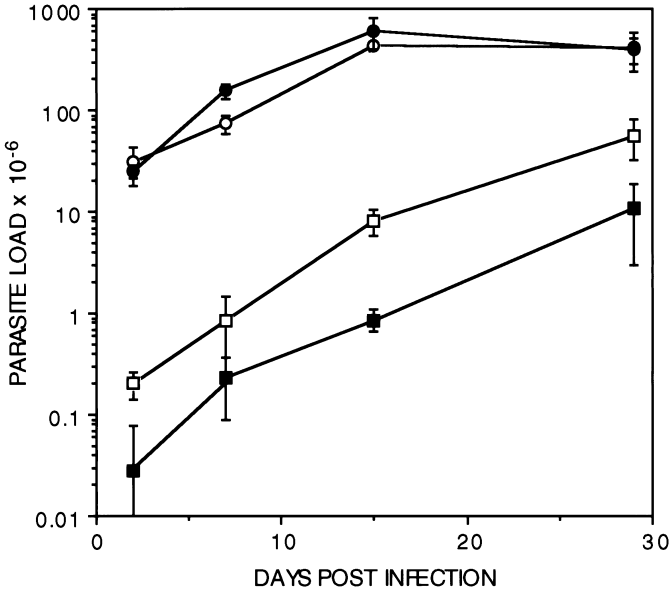

Figure 3. Time-course distribution of *L. infantum* in the spleen (squares) and liver (circles) of BALB/c mouse. Mice were infected intraveneously at $t=0$ with 10^8 promastigotes as described in the Experimental section and organs were taken at the indicated times and analysed by touch imprints (filled symbols) and ELISA (open symbols). Each point represents the average load (mean $\pm$ 1S.D) obtained from 5 different infected mice, bred under the same conditions.

ELISA was set up using another serum sample (Lia) as antibody source. Figure 5 shows that, when spleen and liver samples from infected mice were quantitated by both ELISAs, close values were obtained using two different human antibodies.

DISCUSSION

In studies on visceral leishmaniasis using mouse or hamster experimental models, the enumeration of parasites in various target organs is a parameter of crucial importance.(4) This statement aalso holds true in cutaneous leishmaniasis, since it has been shown(6) that, in *L. major*- infected BALB/c mouse, the lesion size is not always proportional to the number of parasites present in the lesion. Although sensitive detection or enumeration of parasite in various organs could recently be achieved by limiting dilution

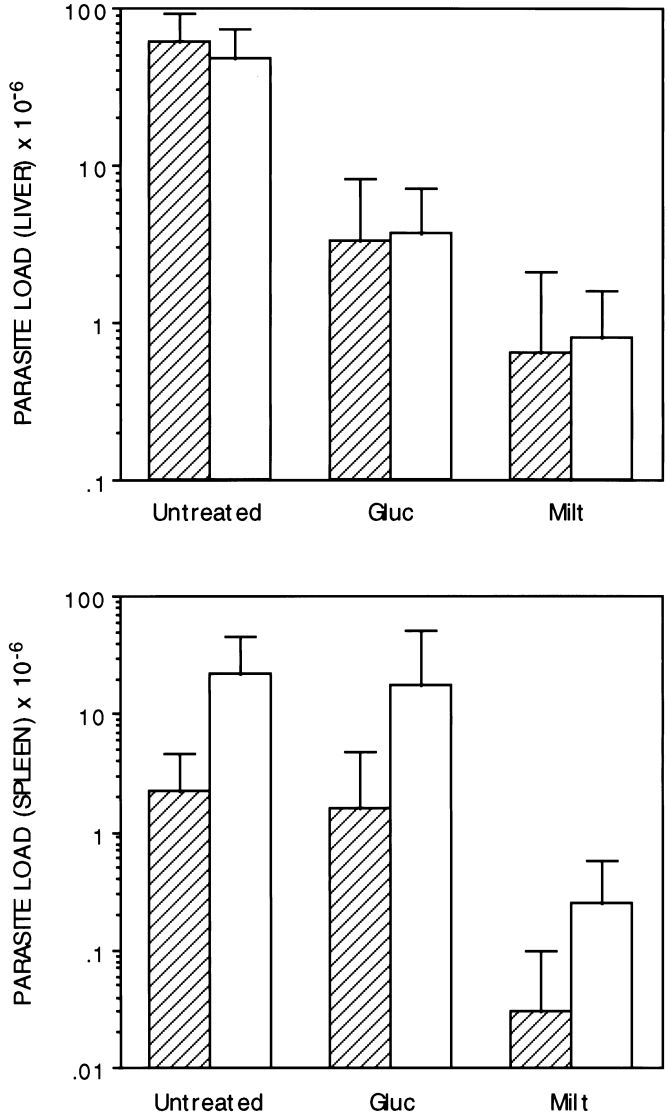

Figure 4. Study of the long-term efficacy of miltefosine against *L. infantum* by touch imprint and ELISA. BALB/c mice were infected on D_0 as described in the Experimental section and treated on D_{28}, for 5 days, with either miltefosine (20 mg/kg body weight/day) or glucantime (200 mg/kg of body weight/day) or left untreated. On day 42, spleen and liver parasite burdens were assessed by touch imprints (hatched bars) and ELISA (open bars). Each parasite load value represents the mean $\pm$ 1 SD for 11 mice.

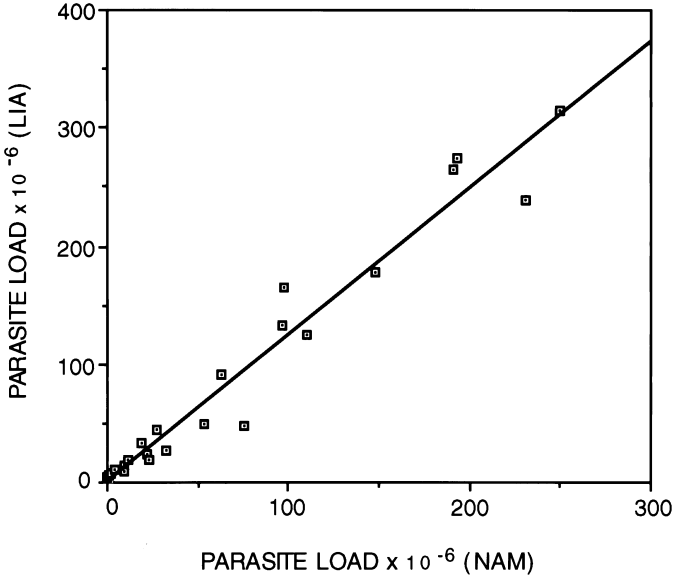

Figure 5. Comparison of ELISAs. 23 liver or spleen samples from infected mice were assessed for their parasite content by the two different ELISAs set up with antibodies from VL patients Nam or Lia and compared. Equation of the regression curve was: $y = 1.24x + 3.31$ and coefficient of correlation was 0.956.

assay, with a theoretical limit of detection of 100 parasites per 100 mg tissue, or by PCR, these techniques suffer from some drawbacks.

For example, in cell culture limiting dilution technique, the *in vitro* transformation of intracellular amastigote into viable promastigote demands, at least, a one-week period of incubation and necessitates the microscopic inspection of numerous dilutions. The PCR technique requires one to obtain good quality DNA, and its quantitative form needs numerous external and internal controls to be reliable. For these reasons, these techniques are not widespread and, in most previous and recent experimental studies, enumeration of parasites in different organs has been achieved by microscopic examination of stained touch imprints, which still represents the reference technique, but requires evaluation by an experienced pathologist, with lengthy searching, using oil immersion microscopic power.

It is noteworthy that the Stauber's formula, which allows to extrapolate the parasite load from the average number of amastigote *per* nucleus, has only been established for liver and, to our knowledge, never validated for other organs. In the present study, parasite loads in *L. infantum*-infected

BALB/c mice were measured by an original and convenient method including first, a solubilization of amastigote-containing tissues with NP40 detergent and, then, the quantitation of solubilized amastigote antigens by sandwich-type ELISA, set up with a high titer human anti-*L. infantum* antiserum. The assay allows, in practice, the specific and precise detection of 5 x 10^4 amastigotes *per* 100 mg tissue, a sensitivity close to that (2 x 10^4/100 mg) published for the imprint technique(5) and representing parasite burdens of 0.05 x 10^6 and 0.5 x 10^6 for a 100 mg spleen and a 1000 mg liver from BALB/c mouse, respectively.

The selection of a polyclonal antibody of human origin could be, at first glance, opened to criticism, but relies on the following grounds. A method designed with monoclonal antibodies would assay only a single protein per parasite and, thus, would certainly result in a considerably decreased sensitivity. In addition, the two different epitopes necessary for a sandwich assay should be simultaneously present and in the same conformational state in both, purified amastigotes used for standardizing the assay, and amastigotes in tissue to be measured. In this respect, a polyclonal antibody which ensures a large recognition of the numerous antigens present in the parasite, as it appears by Western-blot analysis, is *de facto* more specific and capable of providing a high sensitivity method. This statement was confirmed by the close reactivity of amastigote and promastigote observed by ELISA.

Furthermore, in our hands, most, if not all, rabbit anti-*L. infantum* immune sera or sera from infected hamster and mouse, exhibited by ELISA antibody titers lower than those observed for the best human sera, obtained at diagnosis from patients with patent leishmaniasis and several sera, other than Nam showed by ELISA, antibody titers compatible with the development of highly sensitive tests.

Interestingly, the ELISA developed with such a serum (Lia) gave, with mouse organs, results comparable to those obtained with the method presented here, thus strongly suggesting that many sera from VL patients are suitable for developing accurate immunoassay tests. Furthermore, it demonstrated a 10 times higher sensitivity than the method set up with Nam, which could still be 5-fold improved using the biotinyl-tyramide amplification system.(17)

Finally 10 mL of serum from Nam allowed us to prepare reagents for approximately 10^5 determinations and even commercial kits (Vironostika, Organon) for P24 HIV-1 antigen determination have been designed with antibody of human origin.

Spleen and liver parasite loads of BALB/c-infected mice in various experiments(12,13,14) were quantified both by ELISA and touch imprints reference method. In the case of liver, the two methods agreed well with

close absolute values, thus validating the usefulness of the ELISA approach. Conversely, in spleen measurements, although a good agreement was still observed between the two methods, the ELISA values exceeded, by approximately 10-fold, those obtained by imprints. At least a large part of this difference originates from the underestimation brought about by Stauber's formula in the case of spleen.

Indeed, as mentioned above, this formula has been established for liver, assuming a number of 2 x 10^5 cells per mg of tissue. This cell density remains relatively constant throughout the whole infection period, together with a moderate hepatomegaly accompanying the transitory presence of parasite in this target organ.(18) The situation is markedly different in the spleen, where we, and others,(18) observed an important splenomegaly, with organ weight increases currently reaching 8-fold. Moreover, this splenomegaly was, in most cases, accompanied by a decrease in the cell density (unpublished data), from roughly, 12 x 10^5 cells/mg in the spleen from uninfected mouse or mouse with recent infection to 2 x 10^5 cells/mg in the organs presenting an important splenomegaly due to a chronic *L. infantum* infection.

Consequently, in spleen, values obtained with the touch imprint method are largely underestimated and would have to be multiplied by an unpredictable factor ranging from 6 to 1. In this respect, it is remarkable that the ELISA, which provides a direct measure of the parasite number *per* mg organ, automatically takes into account the cell density parameter. Moreover, when spleen touch imprints were histochemically labelled with the antibody selected for the ELISA, parasite counts were, as previously reported,(19) markedly more elevated than those obtained by Giemsa staining (data not shown), suggesting that some atypical form of amastigote, only easily detectable by an antibody-based technique, could be present in spleen and could, therefore, contribute to the difference observed between the two methods.

In addition, some spleen samples, corresponding to low infection states, appeared negative by touch imprints, but were found to contain assayable material by ELISA. This latter finding confirmed that, when parasites are confined to a few infected macrophages, optical quantification is not considered reliable(20) and strongly suggests that the ELISA protocol here presented, based on the analysis of a large piece of the spleen, is more suitable for detecting the low infection states than the touch imprint based on the analysis of around 1000 cells. Taken as a whole, these results indicated that previous studies based on touch imprint underestimated, to a certain degree, the abundance of the parasite in this organ.

Finally, in the various experiments we have performed in the *L. infantum*-infected BALB/c model, the results obtained by ELISA and

touch imprints led to identical conclusions, therefore indicating that the former technique was, in particular, suitable to evaluate the efficiency of leishmanicidal drugs. Therefore, the ELISA presented here represents a valuable alternative method for assessing spleen and liver *L. infantum* infection in various situations. It is particularly well adapted to large series occurring in experimental studies and is useful to detect the low infection states. This technique is likely to be applicable to the enumeration of *L. infantum* or other *Leishmania* species in different tissues, such as lung or bone marrow and, perhaps more generally, to the quantification of various microorganisms.

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