Kinetics and Diagnostic and Prognostic Potential of Quantitative Western Blot Analysis and Antigen-Specific Enzyme-Linked Immunosorbent Assay in Experimental Canine Leishmaniasis

D. Talmi-Frank,1 D. Strauss-Ayali,1,2 C. L. Jaffe,2 and G. Baneth1*

School of Veterinary Medicine, The Hebrew University, P.O. Box 12, Rehovot 76100, Israel,1 and Rabin Medical Centre of the Study of Tropical and Infectious Diseases, Hebrew University-Hadassah Medical School, Jerusalem, Israel2

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Quantitative computerized Western blot analysis of antibody responses during experimental canine *Leishmania infantum* infection distinguished between immunodominant and nonimmunodominant protein bands. Six infected beagles, positive by both PCR and parasite culture, were monitored over 75 weeks postinfection and during a 12-week allopurinol treatment course. All dogs were asymptomatic at the time of treatment. Of 12 antigenic bands examined, the immunodominant bands (12, 14, 24, 29, 48, and 68 kDa) showed significantly increased intensities (P < 0.01) and higher frequencies of recognition than the nonimmunodominant bands at all time points. Detection of the former bands at 6 weeks postinfection preceded seroconversion by enzyme-linked immunosorbent assay (ELISA) both on crude *Leishmania* antigen or the recombinant proteins rK39 and HSP70. Reactivity with the 14-, 48-, and 68-kDa bands signified early infection, whereas increased reactivity with the 14-, 24-, and 29-kDa bands was associated with posttreatment parasite persistence and potential unfavorable prognosis. Total lane intensity (TLI) emerged as a sensitive marker for early infection and increased as early as 4 weeks postinfection. TLI had a significantly higher (P < 0.01) relative increase rate than crude *Leishmania* antigen or HSP70 or rK39 ELISA at all time points. These immunodominant antigens and TLI, as determined by quantitative Western blotting, will be valuable for early detection and treatment evaluation of canine leishmaniasis.

Visceral leishmaniasis caused by *Leishmania infantum* is an important sand fly-borne human and canine disease. Canine visceral leishmaniasis (CVL) is widespread in southern Europe and the Mediterranean basin, and seroprevalence rates may reach 40% in some disease foci (8). Dog exposure to infective sand fly bites can result in an asymptomatic period that may progress to symptomatic infection or remain cryptic. Both symptomatic and asymptomatic dogs harbor the parasite and are infective to sand fly vectors (7, 16). Infected dogs that are medically treated for leishmaniasis often relapse following the end of therapy. Diagnostic efforts and control measures are therefore aimed at early and specific diagnosis of infection and also at the monitoring of dogs following antileishmanial treatment.

Most of the serological techniques currently used to detect CVL are based on reactivity against whole-parasite antigens. These assays detect high antibody levels, whereas early and subclinical infections in dogs that are parasitologically positive are often characterized by low antibody titers (9, 22). Western blot analysis of whole-parasite antigens is considered sensitive when low serum antibody titers are present (25) and has been proven to be a highly specific method for diagnosis of CVL (5). Antibodies to specific recombinant antigens such as heat shock protein 70 (rHSP70) and K39 (rK39) have also been shown to be good markers for infection (17, 27). HSP70 is a target for the humoral response during human leishmaniasis (13) and has been shown to stimulate the murine immune system (23). rK39 is mainly expressed in the amastigote stage and elicits a strong immune response in both asymptomatic and clinically infected dogs (26).

The purpose of this study was to identify early markers for *L. infantum* infection and prognostic factors by monitoring changes in titers to specific proteins before, during, and after therapy against CVL. The serological response to leishmanial antigens was evaluated in experimentally infected dogs using quantitative Western blot analysis and enzyme-linked immunosorbent assay (ELISA).

**MATERIALS AND METHODS**

Experimental infection with *L. infantum*. A group of 6-month-old laboratory-bred male beagle dogs (n = 6) were intravenously inoculated with *L. infantum* amastigotes, as previously described (30). At 32 weeks postinfection (PI), when infection was parasitologically confirmed and seroconversion was evident, treatment with 20 mg/kg of body weight/day allopurinol was administered orally for 3 months. The dogs were monitored for up to 18 months, including a period of 8 months before and 10 months after initiation of treatment. The second group included 6-month-old male *L. infantum*-seronegative beagles (n = 3) that served as noninfected controls and were inoculated with sterile saline at the time of *L. infantum* inoculation of the experimentally infected dogs. This study was approved by the Animal Ethics Committee of the Hebrew University of Jerusalem (MD-64,18-06, DHHS animal welfare assurance number OPRR-A01-5011).

**Serological follow-up**. Sera were collected from the dogs prior to infection and then periodically, at least every month pretreatment, once close to cessation of therapy, and then 6 months later. Samples were stored at −20°C until tested. Anti-*Leishmania*-specific antibodies were examined by Western blot analysis and by ELISA using rHSP70, rK39, and crude antigens.
Temperature (RT). After washing as described previously, 50 μl of serum diluted at 1:100 or 1:200 (for rK30 and rHSP70, respectively) in PBS-T containing 2% FCS was added to each well and incubated at 37°C for 30 min, followed by three washes with PBS-T. Plates were incubated with protein A diluted 1:8,000 (rK39) or horseradish peroxidase-conjugated goat anti-canine immunoglobulin G antibody (Cappel research reagents, ICN Biomedicals, Aurora, OH) diluted 1:5,000 (rHSP70) in PBS-T containing 2% FCS for 30 min at RT. Excess conjugate was removed by extensive washing with PBS-T, and the plates were developed by addition of 2,2'-azinois(3-ethylbenzthiazolinesulfonic acid) (ABTS). The positive- and negative-control sera used for standardization of this assay were the same as used for the crude antigen ELISA. Each plate was read when the absorbance (A ~ 0.05 nm) of the positive reference reached an OD value between 1.8 and 2.0.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis for CLA, rK39, and HSP70 antigens. L. infantum CLA, rK39, and HSP70 antigens were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. CLA was used at 150 μg/ml, while both rK39 and HSP70 were used at 150 ng/ml. Antigens were heated with the sample buffer at 50°C for 5 min and subjected to a 12% polyacrylamide gel under nonreducing conditions (11) using a minielectrophoresis apparatus (Bio-Rad Laboratories, Inc.). The resolved polypeptides were transferred onto a nitrocellulose membrane in Tris-glycine buffer containing 25% methanol at a constant 65 mA for ~45 min at RT. The membranes were cut to individual strips and blocked with PBS containing 0.5% Tween 20 and 5% dried milk for 2 h at RT, followed by five extensive washings with PBS-T. Dog sera diluted 1:100 with PBS-T were incubated with the strips for 2 h. Positive- and negative-control sera were included in each procedure to avoid interassay variations. Nitrocellulose strips were individually washed five times as before and incubated with an horseradish peroxidase-conjugated goat anti-canine immunoglobulin G (Cappel research reagents, ICN Biomedicals, Aurora, OH) diluted 1:5,000 in PBS-T for 1 h at RT. After five extensive washings, the strips were developed with 0.0004% 3,3'-diaminobenzidine (DAIB; Sigma Chemicals, Missouri). A molecular mass protein standard (Precision Plus protein standards; Bio-Rad, Hemel Hempstead, United Kingdom) was included in each procedure.

Quantitative KODAK one-dimensional software analysis. The blotted membranes were scanned with a 6200C scanner (Hewlett-Packard Co., Mississauga, Canada), and the image was analyzed using the KODAK one-dimensional software analysis (Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY) according to parameters defined by the software algorithms for band and lane parameters. Protein bands were digitized, and the net band intensities for each band and total lane intensities were recorded and expressed as pixels. Lane markers and isomolecular mass lines were manually defined, followed by program-generated marking of individual bands. Standardization of band visualization was carried out by setting gamma (γ = 1) as a constant for brightness and contrast. By definition, the gamma value represents the nonlinear relationship between pixel value and lumiance or intensity (20). The bandwidth percentage, defined as the width of the band used for the analysis as a percentage of the total bandwidth, was set to 80%; and the sensitivity level relating to the resolution of the band in comparison to its background was set to the default value of 0 on a scale of −3 to +3. These settings were maintained throughout the analysis. Quantitative analysis of overlapping bands was approximated by fitting the data to a symmetric Gaussian curve (32). Net band intensities were defined as the sum of all valid band intensities in the range of each individual lane.

PCR and Leishmania culture. Spleen and lymph node aspirates were periodically sampled before and after treatment with allopurinol (30). DNA extraction and PCR for a fragment of the internal transcribed spacer 1 of the Leishmania ribosomal operon were carried out as previously described (26). In addition, spleen and lymph node aspirates were cultured for Leishmania parasites in NNN medium as previously described (2).

Statistical analysis. All data comparisons were analyzed for statistical significance with the Statistix analysis software, version 7.0 (Analytical Software, Tallahassee, Florida) using t test for comparison between immunodominant and nonimmunodominant bands and two-way analysis of variance together with the Tukey test for comparisons of means for the rest of the analyses. Differences were considered significant at a P value of <0.05.

RESULTS

Infection status of dogs. All of the L. infantum-inoculated dogs were positive by culture and PCR of spleen and lymph node aspirates 1 month PI and remained positive throughout the infection period, except for 1 dog that was negative at 3 months PI by both PCR and culture and became positive thereafter (30). By 32 weeks PI, five of the experimentally L. infantum-infected dogs developed mild clinical signs and were defined as oligosymptomatic. During the follow-up period after the allopurinol treatment was stopped, all dogs became asymptomatic, except for 1 dog that developed renal failure and was euthanized 22 months PI. Positive PCR was recorded for all dogs during follow-up after cessation of treatment.

ELISA for CLA, rHSP70, and rK39. The kinetics of antibodies to CLA, rHSP70, and rK39 determined by ELISA are shown in Fig. 1. Elevated levels of specific antibodies to HSP70 and rK39 were detected after 6 weeks PI, while antibodies reactive to CLA reached the positive cutoff value (OD, 0.6) at only 8 weeks PI. Reactivity against rHSP70 and rK39 increased significantly faster than the reactivity to CLA between 6 to 8 weeks PI (P < 0.0012), peaking at an OD of 2.25 and 2.05 for rHSP70 and rK39, respectively, by 19 weeks PI, higher than the OD of 1.45 obtained for CLA at the same time point. During and after cessation of treatment, antibodies to rHSP70 dropped at a significantly faster rate than those to CLA and rK39 (P < 0.0007). No seroconversion was found in the 3 control dog sera at any time point when tested with CLA, rHSP70, and rK39 (data not shown).

Western blot analysis. Antibodies from the infected dogs recognized up to 12 bands with molecular masses ranging between 12 and 136 kDa in L. infantum crude antigen by Western blotting. Reaction to each band was assessed according to its mean net intensity and recognition prevalence by each dog's sera at five different time points during the experimental period, including 3 points before and 2 points after treatment.
TABLE 1. Comparison of mean band intensities of immunodominant and nonimmunodominant bands expressed at five different time points during the experimental period

| Band category  | Band (kDa) | Pretreatment Mean intensity ± SEM at wk PI* | Posttreatment Mean intensity ± SEM at wk PI* |
|---------------|-----------|-------------------------------------------|-------------------------------------------|
|               |           | 8                | 14                | 29                | 48                | 75                |
| Immunodominant| 12        | 7.3 ± 30 (5)     | 9.1 ± 3.7 (6)     | 9.6 ± 3.9 (5)     | 12 ± 4 (5)        | 0.2 ± 3.3 (4)     |
|               | 14        | 9.8 ± 4.6 (6)    | 20 ± 8.8 (6)      | 21.4 ± 4.8 (6)    | 35.7 ± 22.3 (6)   | 30.7 ± 18.8 (6)   |
|               | 24        | 17 ± 7.4 (5)     | 13.3 ± 4 (5)      | 8.7 ± 1.66 (6)    | 15.2 ± 5.2 (6)    | 13.4 ± 5.4 (5)    |
|               | 29        | 17 ± 8.1 (5)     | 22.8 ± 6.6 (6)    | 18 ± 3.8 (6)      | 23.9 ± 9.7 (6)    | 22.8 ± 7.2 (6)    |
|               | 48        | 15 ± 5 (3)       | 16.6 ± 5.2 (3)    | 6.7 ± 2.7 (4)     | 5.8 ± 3.0 (3)     | 10.0 ± 4.7 (2)    |
|               | 68        | 16.5 ± 0.15 (4)  | 13.1 ± 1.9 (4)    | 15.5 ± 1.4 (4)    | 14.0 ± 0.1 (4)    | 14.6 ± 0.5 (4)    |
| Nonimmunodominant| 18       | 7.6 ± 0.5 (2)    | 6.6 ± 1.6 (5)     | 4.1 ± 1.6 (3)     | 12.0 ± 8.4 (3)    | 5.1 ± 2.3 (4)     |
|               | 19        | 0.8 (1)          | 8.4 ± 2.6 (4)     | 4.2 ± 1.4 (3)     | 1.2 (1)           | 5.4 (1)           |
|               | 34–35     | 8 ± 5 (3)        | 8.4 ± 2.6 (5)     | 3.9 ± 1.7 (4)     | 2.5 ± 1.1 (3)     | 1.3 (1)           |
|               | 71        | 12.8 ± 5.2 (2)   | 10.2 ± 3.5 (2)    | 9.4 ± 1.9 (2)     | 13.1 ± 2.4 (2)    | 11.0 ± 0.4 (2)    |
|               | 102       | 12 ± 8.3 (3)     | 8.4 ± 5.4 (3)     | 5.4 (1)           | 7.3 (1)           | 7.3 (1)           |
|               | 136       | 3 ± 1 (5)        | 4.8 ± 0.1 (5)     | 0                 | 0                 | 0                 |

* Mean intensity was determined at 10^6 pixels. Values in parentheses indicate numbers of dogs expressing bands.

(31) No reactivity with the 3 control dog sera was found at any time point.

Classification to immunodominant and nonimmunodominant bands. The net intensity of the measured bands ranged from 0.2 × 10^6 to 35.7 × 10^6 pixels. Mean band intensities and standard error data are shown in Table 1. Six bands, 12, 14, 24, 29, 48, and 68 kDa, had significantly higher net intensities (P < 0.01) than the other bands, 18, 19, 34 to 35, 71, 102, and 136 kDa, at all time points. In addition, the recognition prevalence of the 6 former bands among the infected dogs was usually higher than that of the latter bands. According to these criteria, bands of 12, 14, 24, 29, 48, and 68 kDa were defined as being immunodominant, whereas the remaining bands were classified as nonimmunodominant.

Patterns of band detection. Longitudinal analysis of the 24-, 48-, and 68-kDa bands (Fig. 2) showed that reactions with these bands were already elevated at 4 to 6 weeks PI, approximately 2 to 4 weeks earlier than the CLA, rHSP70, and rK39 ELISA. All 3 bands reached a peak of intensity between 6 and 8 weeks PI and then slowly declined over 8 to 25 weeks PI. During the course of treatment, reaction against the 48-kDa band declined rapidly, while reaction against the 24-kDa band intensified. Reaction against the 12-, 14-, and 29-kDa immunodominant bands, while weaker than that found for the 24-, 48-, and 68-kDa bands slowly increased with time PI (Fig. 3). The former bands were initially recognized by 4 to 6 weeks PI, showing a relatively similar reaction pattern consisting of a considerable increase in intensity at 6 weeks PI and preceding CLA, rK39, and rHSP70 ELISA seroconversion. Band intensities reached a peak between 21 to 25 weeks PI, lowered before the beginning of treatment, then reintensified at 48 weeks PI, and dropped again 75 weeks PI after cessation of treatment. The relative increase rate was calculated as the ratio between the values of a parameter at a given time and preinfection (time zero). A comparison between the relative increase rate of the 14- and 29-kDa bands to CLA ELISA
showed significantly higher (P < 0.0006) relative increase rate for the 14-kDa band than for the CLA between 14 to 21 weeks. The 29-kDa band showed a significantly higher (P < 0.01) relative increase rate between 8 and 21 weeks.

The 34- to 35-kDa band had a unique reactivity pattern. It initially appeared at 6 weeks PI, reached relatively low levels of intensity, which remained stable for 5 months, and decreased at the end of the experiment (data not included in figures). The 136-kDa band appeared at 2 to 4 weeks PI, reached a peak 11 weeks PI, and disappeared as early as 19 weeks PI. The additional nonimmunodominant bands of 18, 19, 71, and 102 kDa had a low frequency of detection and were not included in the figures.

**Western blot analysis for rK39 and HSP70.** Western blot analysis for antibody reaction to rK39 and HSP70 antigens was evaluated at preinfection (time zero) and at 2, 4, and 6 weeks PI using the same analytical settings as for the CLA. This was done to evaluate the time of appearance of reactivity to these antigens by Western blot analysis and to compare it to ELISA. Reactivity with rK39 and HSP70 was detected only at 6 weeks PI and not earlier, in agreement with the ELISA results for these antigens.

**TLI.** The relative increase rate, e.g., the ratio between the pixel value at a certain time point and preinfection (time zero), was compared between rK39, rHSP70, CLA ELISA, and TLI (Fig. 4). It showed a significantly higher (P < 0.01) relative increase rate of TLI at all time points starting as early as 4 weeks PI. The intensity of TLI increased after treatment, in contrast to all ELISA assays, which showed decreases in optical density.

**DISCUSSION**

The present study describes the first longitudinal quantification of serologic reactivity to *L. infantum* antigens by Western blotting in an experimental canine infection. Computerized quantification of band intensity was able to clearly distinguish between immunodominant and nonimmunodominant bands. This statistically significant discrimination of band intensities into immunodominant and nonimmunodominant categories was reproducible at all time points. Recording of the net intensity for each band allowed one to follow up the changes in reactivity to individual bands with time. In addition, quantification of the entire blot lane intensity, defined as TLI, a parameter that has not been used in previous similar studies, proved to be a highly sensitive indicator of infection and post-treatment prognosis.

Seroreactivity with low-molecular-mass bands emerged as a valuable marker for early and persistent infection in this study. The 12- and 14-kDa immunodominant bands appeared as early as 4 to 6 weeks PI and expressed high intensities throughout the follow-up period of 75 weeks. This is in agreement with findings from naturally infected dogs, where these 2 bands were recognized early by antibodies in asymptomatic dogs that eventually seroconverted during a longitudinal study (1). Similar to our findings in this study, these 2 bands were recognized by sera from naturally infected dogs prior to seroconversion in a dot ELISA using whole-parasite antigen (1). Studies of human immunodeficiency virus-*Leishmania*-coinfected humans and of asymptptomatically infected people in the Mediterranean basin have shown that antibodies which recognize 14- and 16-kDa *Leishmania* antigens were very sensitive indicators of parasite infection (4, 12, 14).

The 24-, 29-, 48-, and 68-kDa bands represent additional immunodominant polypeptides whose recognition preceded both CLA and the recombinant rK39 and rHSP70 antigens. Reactivity with these bands appeared as early as 4 to 6 weeks PI. The strongest reaction was seen against the 48- and 68-kDa bands, emphasizing their potential importance as sentinels of early infection. Reactions with the 14-kDa band reached higher intensities than the 24-kDa band at 6 to 14 weeks PI and were also considered a good candidate marker for early infection. Other studies in naturally infected dogs have described reactions against closely located midscale bands, such as the 28-, 46-, and 68-kDa bands in asymptomatic dogs or the 29-, 50-, and 67-kDa bands in symptomatic dogs (6).

Animals treated with allopurinol in the current study showed clinical recovery and became asymptomatic but remained parasitologically positive. Clinical recovery was not accompanied by disappearance or waning of reactivity to the 14-, 24-, and 29-kDa immunodominant bands, contrary to what has been previously reported for the 29-kDa band (31). Rather, the intensity of reaction with these bands remained strong and stable. This might be explained by destruction of amastigotes and increased exposure to antigens released from dead parasites during treatment. However, the presence of high band intensity at 27 weeks posttherapy indicates that the level of reactivity with these bands might serve to mark treatment failure and the persistence of parasite in visceral tissues.

*L. infantum* promastigote antigens are not identical to all antigens expressed by amastigotes. However, many cross-reactive antigens are present in the two parasite stages, and promastigote antigens have been used in most CVL Western blot studies (1, 5, 9, 31). The identities of the immunodominant polypeptides detected by Western blotting are largely un-
known. Proteomic analysis and characterization of the immunodominant membrane-associated fraction of *L. infantum* antigens reacting with sera from Mediterranean visceral leishmaniasis patients (10) identified a 50-kDa band. This protein showed good homology to *L. donovani* elongation factor 1α (EF-1α), which participates in protein synthesis in eukaryotic cells (3). This protein is also involved in apoptosis regulation and is essential for ubiquitin-dependent degradation (19). EF-1 consists of two functionally distinct parts, EF-1α and EF-1γ, previously mentioned as EF-1 subunits α, β, and γ, having molecular masses of 50, 30, and 48 kDa, respectively (3). The immunodominant 48-kDa band found in the present study may correspond to the 50-kDa protein identified by Kamoun-Essghaier et al. (10), homologous with *L. donovani* EF-1α. This could explain the sharp decrease in the reactivity to the 48-kDa band during treatment and support the assumption that this band is an indicator of early infection to activate multiplication of parasites and increased protein synthesis in recently infected individuals.

HSP70 is a member of the heat shock protein family and has been shown to act as a mitogen for murine B cells (24). Recombinant K39 is a repetitive immunodominant epitope in a kinesin-related protein that is highly conserved among viscerotropic Leishmania species (27). Both of these proteins have been proposed as markers for early infection and as highly sensitive and specific diagnostic antigens for human visceral leishmaniasis (21, 29) and CVL (15, 17, 18, 27). The present study showed that, although antibodies against these two antigens became markedly elevated during the experimental infection, the relative increase for CLA ELISA was greater (Fig. 4). Even more surprising, we found that the relative increase in the TLI was even greater and surpassed all of the ELISAs tested. Thus, the quantification of the TLI emerged as a most sensitive indicator for infection with *L. infantum*, and its application for disease diagnosis needs to be investigated further.

In summary, quantitative analysis of Western blotting proved to be a useful method for identifying diagnostic antigens for further study. Antibody responses during experimental canine *L. infantum* infection were characterized by reactivity to the 12-, 14-, 24-, 29-, 48-, and 68-kDa immunodominant leishmanial proteins. Reactivity with the 14-, 48-, and 68-kDa bands appeared to be good markers for early infection, whereas increased reactivity with the 14-, 24-, and 29-kDa bands following allopurinol treatment was associated with parasite persistence and potential unfavorable prognosis. TLI elevation could be used as a marker of both early infection and posttreatment parasite persistence. Future studies focusing on the characterization of individual antigens will facilitate better understanding of the host immune response to specific proteins and contribute further to the development of diagnostic tools and treatment targets. This method should also prove helpful in understanding the kinetics of immune responses against pathogens in other infectious diseases.

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