

Kinetics of Canine Antibody Response to Saliva of the Sand Fly *Lutzomyia longipalpis*

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ABSTRACT

Zoonotic visceral leishmaniasis (VL) caused by *Leishmania infantum* is transmitted from dogs to humans by sand flies and *Lutzomyia longipalpis* is a major vector of this disease. We studied the antibody response in dogs experimentally exposed to *L. longipalpis* females to characterize sand fly salivary antigens recognized by canine sera and to find out whether the level of specific anti-saliva antibodies reflects the intensity of exposure. Sera from repeatedly bitten dogs revealed up to six salivary protein bands with approximate molecular weight of 66, 55, 45, 37–39, 34, and 25 kDa in *L. longipalpis* salivary gland lysate. Anti-saliva immunoglobulin (Ig) G and its subclasses were found to be useful markers of exposure to sand flies. Specific IgG, IgG1, and IgG2 were related to numbers of bloodfed *L. longipalpis* females, and increased antibody levels were detectable throughout the study, i.e. more than 6 months after the last exposure. In contrast, specific IgE response developed in some dogs only, and no correlation was observed between its level and the intensity of exposure. Screening of dog sera for specific IgG against salivary antigens of the vector is suggested as a useful epidemiological tool in VL foci. Monitoring canine antibody response to sand fly saliva also allows evaluation of the effectiveness of anti-vector campaigns. **Key Words:** Immunology—Leishmania—Parasitology—Sand fly (flies)

INTRODUCTION

DOGS AND WILD CANIDS are the natural reservoirs of human visceral leishmaniasis (VL) caused by *Leishmania infantum* (syn. *L. chagasi*). This life-threatening disease occurs in Latin America, Asia, Africa, and Europe. *Leishmania* parasites are transmitted by the bite of an infected sand fly (Diptera: Phlebotominae), and the establishment of infection in naive hosts is promoted by the immunomodulatory effect of sand fly saliva (reviewed by Kamhawi 2000, Rohousova and Volf 2006). At the same time, repeated exposure to sand fly bites elicits an immune response that confers partial protection against naturally transmitted *L. major* in mice (Kamhawi et al. 2000).

Anti-saliva sensitization in hosts bitten by

sand flies employs both arms of immunity (Baral et al. 2000). Although cellular rather than humoral immunity is supposed to be responsible for the protective status against leishmanioses (Valenzuela et al. 2001), the study of anti-saliva antibodies is important for characterization of the immune status of the host. Very little is known, however, about the kinetics of anti-saliva immunoglobulin production in hosts bitten by bloodfeeding insects; few data are available from mice experimentally exposed to *Triatoma infestans* (Volf et al. 1993) or humans naturally exposed to mosquitoes for prolonged periods (Peng et al. 2004). To our knowledge, there is no study describing antibody response kinetics in animals exposed to sand flies.

Anti-saliva antibodies developed by hosts

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bitten by sand flies may provide important epidemiological information. Data from humans living in a focus of anthroponotic cutaneous leishmaniasis in Turkey (Rohousova et al. 2005) suggest that the specific antibody response to sand fly saliva, in this case *Phlebotomus* spp., could be used to monitor the exposure of hosts to sand flies and might also be useful as a marker of risk for *Leishmania* transmission in endemic areas. Moreover, control programs for human VL caused by *L. infantum* are targeted at dogs, the main reservoirs. Prevention of sand fly bites by insecticide treatment is the main way to stop the circulation of canine leishmaniasis (reviewed by Gramiccia and Gradoni 2005, Lainson and Rangel 2005); therefore monitoring canine antibody response to vector saliva would allow evaluation of the effectiveness of anti-vector campaigns.

The main objective of the present study was to define the long-term kinetics of anti-saliva antibody response in dogs bitten by *Lutzomyia longipalpis*. This sand fly is the only proven vector of *L. infantum* in Latin America (reviewed by Killick-Kendrick 1999, Lainson and Rangel 2005). Antibody response was monitored in dogs exposed to low or high numbers of sand fly females to determine whether the level of anti-saliva antibodies reflects the intensity of exposure to sand flies. In addition, we sought to determine the number and approximate molecular weight of *L. longipalpis* salivary antigens reacting with dog IgG antibodies.

MATERIALS AND METHODS

Dogs and sand flies

Twelve adult laboratory beagle dogs were housed in the animal facility of Bayer Health-Care AG (Leverkusen, Germany). They were maintained and handled in accordance with European guidelines for ectoparasiticide efficacy studies. Sand flies of *L. longipalpis* colony (originating from Jacobina, Brazil) were reared in standard conditions as described by Benkova and Volf (2007). Salivary glands were dissected from 3–6-day-old females. Groups of 20 glands in 20 μ L of 20 mM Tris buffer with 150mM NaCl (TBS) were stored at -70°C . Prior to use, salivary gland lysate (SGL) was prepared by re-

peated freezing and thawing as described elsewhere (Volf and Rohousova 2001).

Exposure to sand flies and blood collection

Dogs were sedated with medetomidine at a dosage of 1.0 mg active ingredient (a.i.)/m² body surface, and diazepam, 5 mg a.i./dog. Dogs were individually exposed to approximately 200 sand fly females as described by Mencke et al. (2003), once a week for a total of five exposures. After exposure, sand fly females were collected and microscopically examined to assess the feeding success of the fly.

For the main experiment, two groups of three dogs each (groups 1 and 2) were used. Dogs in group 2 were treated with a repellent prior to first exposure and, as a result, were by far less exposed to sand fly bites than dogs in group 1. Therefore dogs in group 1 are defined as high-exposed; dogs in group 2, as low-exposed. Blood samples were collected from vena cephalica antebrachii and processed to serum. Dogs were sampled according the following schedule: pre-immune serum in week 0, immune serum 6 days after each exposure (weeks 1, 2, 3, 4, and 5) and then weeks 6, 7, 9, 13, 19, and 23.

Because elevated levels of anti-saliva IgG were observed even 19 weeks post-exposure, an additional experiment was designed to follow the tapering of specific antibody response. Six beagle dogs (groups 3 and 4) were immunized according to the same exposure protocol, and dogs in group 3 were untreated (high-exposed) and those in group 4 were treated with a repellent (low-exposed). In this experiment, blood samples were collected in weeks 22, 28 and 33.

Measurement of specific IgG and IgG subclasses

Specific anti-saliva IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Microtiter plate wells (CovaLinkTM NH, Nunc) were coated with salivary gland lysate (60 ng of protein per well in 50 mM carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C . The wells were then washed in 20 mM phosphate-buffered saline (150 mM NaCl, pH 7.4) with 0.05% Tween 20 (PBS-Tw) and incubated with 6% horse serum in PBS-Tw for

45 min at 37°C to block free binding sites. Dog sera were diluted 1:500 in 3% horse serum/PBS-Tw and incubated in duplicate for 90 min at 37°C. This step was followed by incubation with peroxidase-conjugated sheep anti-dog IgG (heavy chain specific), goat anti-dog IgG1, or sheep anti-dog IgG2 antibodies (Bethyl Laboratories) at a 1:3,000 dilution for 45 min at 37°C. Orthophenylenediamine and H₂O₂ in McIlwain phosphate-citrate buffer (pH 5.5) were used as a substrate solution. Absorbance was measured with a Multiskan RC ELISA reader (Labsystems) at 492 nm wavelength.

Measurement of specific IgE

Modification of the IgG ELISA protocol was used to measure specific anti-*L. longipalpis* IgE in dog sera. Wells were coated with SGL (180 ng of protein per well), washed in 50 mM Tris buffer (140 mM NaCl, pH 8.0) with 0.05% Tween 20 (TBS-Tw), and blocked with 1% bovine serum albumin (BSA) in TBS-Tw solution for 1 h at room temperature. Dog serum samples were diluted 1:100 in blocking solution and incubated for 1 h at room temperature. The wells were then incubated with goat peroxidase-conjugated anti-dog IgE antibody, ϵ -chain specific (Bethyl Laboratories), at a 1:1,000 dilution for 1 h at room temperature.

Western blot analysis

Salivary gland lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (2.5% 2-mercaptoethanol in sample buffer) in a Mini-Protean III apparatus (BioRad). In preliminary experiments, SGL proteins were separated on 13% gels and immunoblotted. No serum samples reacted with low molecular weight proteins. Therefore, 10% gel was used in further experiments to achieve better resolution of proteins of interest. Separated proteins were stained by Coomassie blue or were electrotransferred onto a nitrocellulose (NC) membrane by Semi-Phor equipment (Hoefer Scientific Instruments). For Coomassie staining, an equivalent of 10 glands (approximately 2 μ g of protein) was loaded per lane. For Western blotting, the protein concentration

per lane was slightly lower (approximately 1.5 μ g). After transfer, the NC membrane was cut into strips and blocked by 5% low-fat dried milk in TBS-Tw for 1 h. The strips were incubated with selected dog sera diluted 1:75 in TBS-Tw for 1 h, followed by 1 h incubation with horseradish peroxidase-conjugated sheep anti-dog IgG antibody (Bethyl Laboratories, dilution 1:3,000 in TBS-Tw). The color reaction was developed using Tris buffer with diaminobenzidine and H₂O₂.

Statistical analysis

Data obtained by ELISA were transformed and subjected to GLM ANOVA (analysis of variance) and Scheffe's Multiple Comparison procedure to analyze statistical significance between means of high- and low-exposed groups at all sampling points. The Bonferroni Versus Control Multiple Comparison procedure was used to test for statistically significant differences between mean antibody levels in pre-immune sera versus immune sera in individual groups. The *p* value was set at 0.05. All statistical analyses were performed with NCSS 6.0.21 software.

RESULTS

Exposure of dogs to sand flies

Beagle dogs (four groups of three dogs each) were individually exposed to approximately 200 *L. longipalpis* females once a week for 5 consecutive weeks. Substantially higher numbers of sand fly females fed on untreated dogs (groups 1, 3) than on repellent-treated dogs (groups 2, 4) (Table 1).

TABLE 1. EXPOSURE OF DOGS TO SAND FLY BITES

Week	Group 1	Group 2	Group 3	Group 4
0	150 $\pm$ 3	3 $\pm$ 2	95 $\pm$ 13	1 $\pm$ 1
1	212 $\pm$ 3	22 $\pm$ 2	113 $\pm$ 15	1 $\pm$ 0
2	141 $\pm$ 11	27 $\pm$ 8	145 $\pm$ 12	10 $\pm$ 5
3	125 $\pm$ 28	24 $\pm$ 7	154 $\pm$ 23	7 $\pm$ 2
4	142 $\pm$ 9	77 $\pm$ 5	134 $\pm$ 12	51 $\pm$ 12

Beagle dogs (four groups of three dogs each) were exposed to *Lutzomyia longipalpis* females weekly five times. Blood-engorged sand fly females were counted after the exposure. Data are represented as mean $\pm$ SEM.

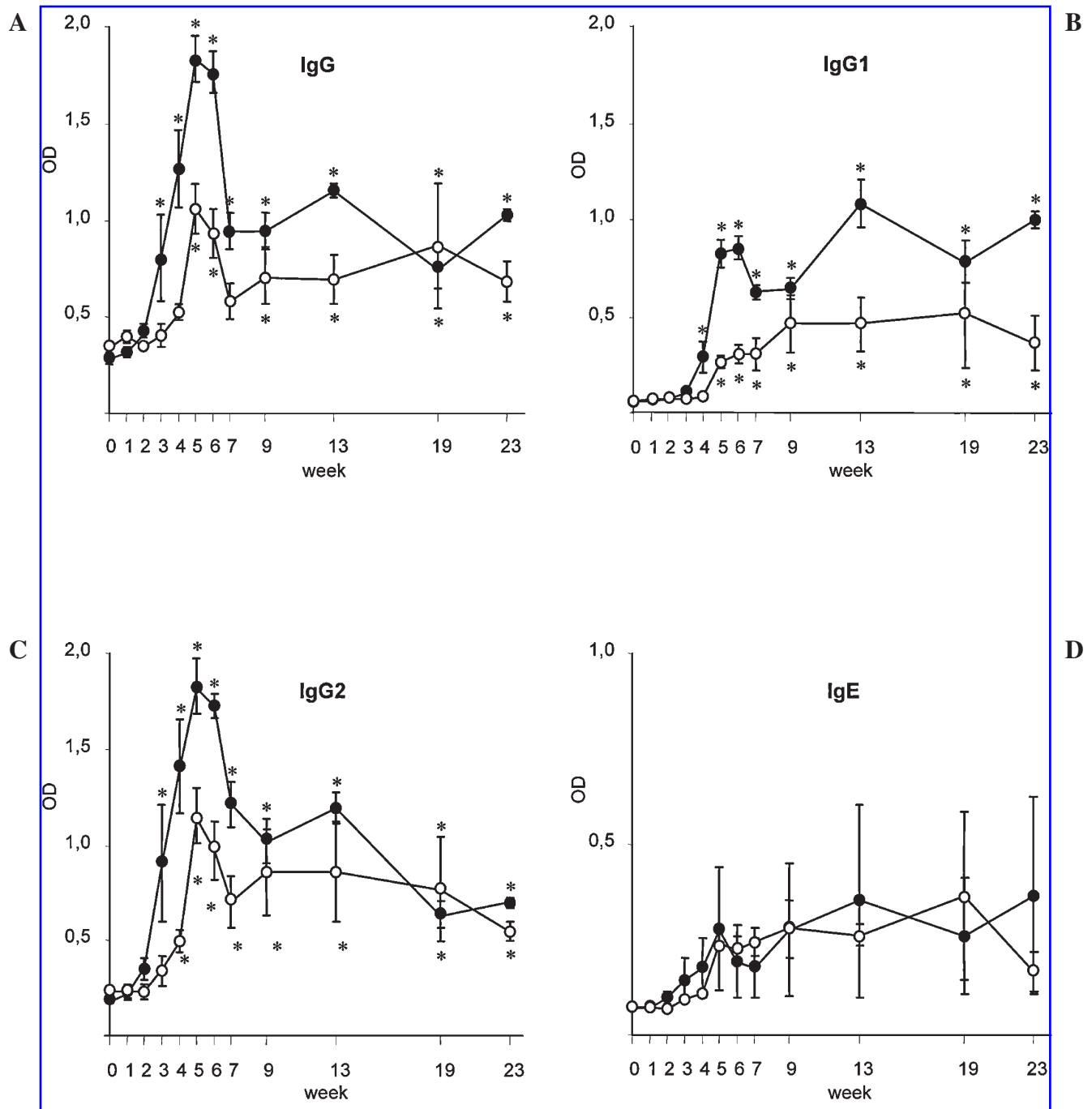


FIG. 1. Anti-*Lutzomyia longipalpis* antibody response in high- and low-exposed dogs. Dogs were immunized by sand fly feeding in weeks 0–4. Specific anti-saliva antibodies were detected by ELISA in pre-immune and immune sera. Full circles, high-exposed dogs (group 1); open circles, low-exposed dogs (group 2); vertical bars, SEM; *Significant difference compared to pre-immune sera ($p < 0.05$)

Kinetics of specific IgG and IgG subclasses

Repeated exposure to *L. longipalpis* bites resulted in high levels of specific IgG to sand fly saliva. No specific antibodies were detected in pre-immune sera of any dog tested.

In three high-exposed dogs (group 1), IgG and IgG2 production showed distinct sharp peaks after the last exposure in weeks 5–6. The trend of IgG1 is different in that, overall, it seems to rise up steadily to week 23. Compared to pre-immune sera, IgG, IgG1, and IgG2 lev-

TABLE 2. SPECIFIC ANTI-*L. longipalpis* SALIVA ANTIBODY RESPONSE IN HIGH- AND LOW-EXPOSED DOGS

	week	IgG	IgG1	IgG2	IgE
group 3	22	0.523 $\pm$ 0.024*	0.474 $\pm$ 0.113	0.478 $\pm$ 0.025*	0.049 $\pm$ 0.134
	28	0.422 $\pm$ 0.036*	0.320 $\pm$ 0.075	0.387 $\pm$ 0.042*	0.527 $\pm$ 0.159
	33	0.471 $\pm$ 0.038*	0.390 $\pm$ 0.093	0.419 $\pm$ 0.058*	0.840 $\pm$ 0.396
group 4	22	0.282 $\pm$ 0.024	0.161 $\pm$ 0.017	0.237 $\pm$ 0.035	0.114 $\pm$ 0.060
	28	0.192 $\pm$ 0.015	0.095 $\pm$ 0.011	0.181 $\pm$ 0.026	0.092 $\pm$ 0.022
	33	0.259 $\pm$ 0.019	0.155 $\pm$ 0.027	0.233 $\pm$ 0.019	0.099 $\pm$ 0.031

Immunoglobulin levels were measured by ELISA in serum samples from beagle dogs. The dogs were exposed to high (group 3) or low (group 4) numbers of *L. longipalpis* females in weeks 0, 1, 2, 3, and 4. Serum samples were taken in weeks 22, 28, and 33. Data are represented as mean optical density $\pm$ SEM. *Significant difference between group 3 and 4 ($p < 0.05$).

els remained significantly elevated until week 23 (i.e., 19 weeks after the last exposure) (Fig. 1A–C).

In low-exposed dogs (group 2), anti-*L. longipalpis* IgG, IgG1, and IgG2 developed with similar kinetics, with overall lower antibody titers. Significant differences ($p < 0.05$) between IgG responses in the high- and low-exposed groups were detected from week 3 onward, with the exception of week 19. The difference in IgG1 responses was significant from week 4 onward; IgG2 responses differed significantly in weeks 3–7. Importantly, even in low-exposed dogs the levels of IgG and IgG subclasses remained significantly elevated compared to pre-immune sera as late as 19 weeks after the last exposure (Fig. 1A–C).

In an additional experiment aimed at tapering of specific antibody response, six dogs in groups 3 and 4 were blood-sampled in weeks 22, 28, and 33. Three high-exposed dogs (group 3) had significantly stronger anti-*L. longipalpis* IgG and IgG2 response than the three low-exposed dogs at all time points ($p < 0.05$). The variance in IgG1 levels was high among individual dogs in group 3; therefore no statistically significant difference was observed between high- and low-exposed dogs (Table 2).

Kinetics of specific IgE

No specific IgE antibodies were detected in canine pre-immune sera. In sera of immunized dogs, variable levels of anti-saliva IgE were observed that could not be related to sand fly exposure intensity. In high-exposed group 1, only one dog developed specific IgE. In low-exposed group 2, all dogs had detectable anti-

L. longipalpis IgE, peaking at different time points (Fig. 1D).

Characterization of *L. longipalpis* salivary antigens

For characterization of *L. longipalpis* salivary antigens recognized by canine sera, serum samples from dogs of group 1 were used. No reaction was observed in control strips where pre-immune sera were used. The antigens recognized by IgG were similar in all dogs, but some differences were observed in time points at which antibodies bound to particular antigens. In all dogs, the reaction was most intense at week 6, i.e., 1 week after the last exposure to sand fly bites. An antigen with a molecular mass of about 66 kDa reacted with sera of all dogs tested; the other bands recognized by sera of exposed dogs had approximate molecular masses of 55, 45, 39, 37, 34, and 25 kDa (Fig. 2).

DISCUSSION

The aim of the present study was to evaluate the kinetics of anti-*L. longipalpis* saliva antibody production in two study groups of low- and high-exposed dogs. The results show that anti-saliva IgG response in dogs bitten by *L. longipalpis* reflects the intensity of exposure. Stronger IgG, IgG1, and IgG2 responses to salivary antigens were observed in high-exposed dogs. The difference in IgG production between high- and low-exposed dogs could be detected as late as 29 weeks after the last exposure to sand flies. This finding may have important consequences for the immune status of

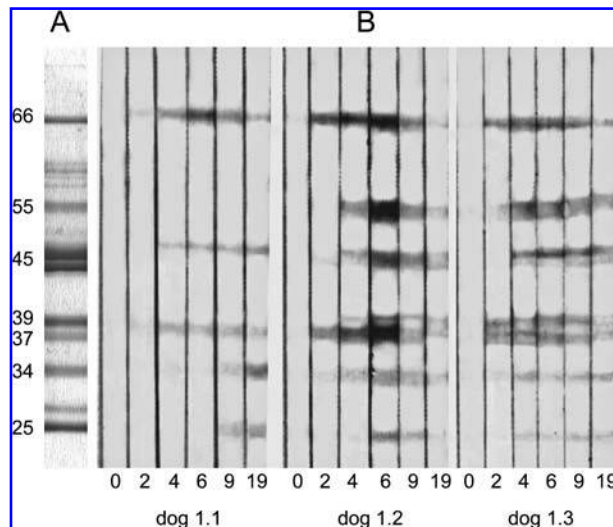


FIG. 2. Characterization of *L. longipalpis* salivary antigens. SGL proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 10% gel. Approximate molecular masses (kDa) indicated on the left. (A) Total protein profile, Coomassie-stained gel. (B) Western blot analysis, nitrocellulose membrane incubated with sera of three dogs repeatedly bitten by *L. longipalpis*. Dogs of group 1 exposed to sand fly feeding in weeks 0–4; sera collected in week 0, 2, 4, 6, 9, and 19.

hosts in natural conditions. In many foci of leishmanioses, sand fly populations show seasonal fluctuations, and the sand fly-free periods last up to 6 months (Lewis and Ward 1987). For example, a strong *L. longipalpis* abundance fluctuation was observed in a Brazilian focus of VL, with sand flies absent during the dry season (de Resende et al. 2006). Our results suggest that anti-saliva antibodies elicited by sand fly bites can persist until the next sand fly season.

The correlation between intensity of exposure to sand flies and host IgG response has implications for insecticide efficacy assessment. As the importance of asymptomatic dogs in the epidemiology of leishmaniosis has been proved by transmission experiments using *P. perniciosus* and *L. longipalpis* sand flies (Molina et al. 1994, Michalsky et al. 2007), treatment of clinically ill dogs or culling of seropositive animals would not be effective. In contrast, topical insecticide application or insecticide-impregnated collars are a good means of preventing *L. infantum* transmission to dogs (Manzillo et al. 2006, Otranto et al. 2007). Mon-

itoring anti-saliva IgG response in combination with infection incidence would provide valuable information on control program effectiveness.

Canine IgG, like IgG of other mammalian species, exists in subclass variants. Four IgG subclasses found in dogs have not been functionally characterized, although associations of specific subclass responses with canine pathologies have been described (Day 1996, Hou et al. 2006). Data available in the recent literature do not support the view that elevated levels of anti-saliva antibodies of either IgG subclass directly affect *Leishmania* infection. In the sera of healthy dogs, IgG1 and IgG2 are the most abundant subclasses, and they occur in similar concentrations (Mazza et al. 1994). Discussion is ongoing about the use of monoclonal antibodies with better-validated specificity (Day 2007) and commercially available polyclonal antibodies.

In dogs exposed to *L. longipalpis* we observed a significant increase in both IgG1 and IgG2 specific for sand fly salivary antigens. The IgG and IgG2 response kinetics developed with a similar pattern, i.e., with a single peak just after the last exposure. In contrast, IgG1 response peaked several weeks post-exposure. This difference may indicate that specific IgG1 production and IgG2 production are regulated in different ways. Although the exact affinities of polyclonal anti-canine antibody conjugates were not known and individual subclass responses could not be precisely compared, IgG2 response was seemingly stronger than IgG1 response.

In contrast to IgG, the kinetics of IgE response were diverse, and not all dogs in the study had detectable specific anti-saliva IgE antibodies. Thus, no significant differences were found in anti-saliva IgE response between high- and low-exposed groups. Similar results were reported by Gomes et al. (2002) for humans; high variation in anti-*L. longipalpis* IgE was found in children who lived in the study area and who, presumably, were equally exposed to sand flies. Taken together, the level of saliva-specific IgE antibodies is not a good marker of exposure.

Sera of dogs exposed to *L. longipalpis* feeding recognized up to six salivary protein bands.

Molecular weight of some of the antigenic bands corresponds to salivary proteins identified previously (e.g., Valenzuela et al. 2004), and we may thus speculate on their identity. The 45 kDa and 37–39 kDa proteins most likely represent members of the Yellow protein family (NCBI accession numbers AF132518, DQ192488). Presumably, the 34 kDa antigen is the apyrase (NCBI accession number AF131933), and the 25 kDa antigen is the D7-related protein (NCBI accession number AF420274). Homologs of some of these proteins were previously reported as antigens or allergens in saliva of bloodsucking arthropods; D7 proteins were shown to be involved in mosquito hypersensitivity (Peng et al. 1998). Sand fly Yellow protein and D7 protein were identified by mass spectrometry as antigens recognized by sera of dogs naturally infected with *L. infantum* (Bahia et al. 2007). In addition, sand fly Yellow protein, D7 protein, and apyrase were previously proposed as antigens recognized by sera of experimentally bitten mice and naturally exposed humans (Gomes et al. 2002, Rohousova et al. 2005). In agreement with our previous results in mice (Rohousova et al. 2005), no IgG binding to peptides or low molecular weight proteins was observed.

In conclusion, we have shown that dogs experimentally exposed to *L. longipalpis* develop specific IgG and IgE antibodies. The levels of IgG and its subclasses were related to number of fed *L. longipalpis* females, and differences in antibody levels between the high- and low-exposed dogs were detectable up to 29 weeks after the last exposure. The study showed that serum anti-saliva IgG is a useful marker of exposure, and that screening of dogs for antibodies specific to *L. longipalpis* salivary proteins should be of major importance in foci of VL caused by *L. infantum*.

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