Passive transmission of humoral and cellular immunity in canine visceral leishmaniasis

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SUMMARY

Visceral leishmaniasis is becoming more frequent in many parts of the world. Where it is endemic, dogs are considered the primary reservoir. Serologic survey of canine populations is one of the measures used to identify positive animals to be sacrificed as a method of disease control. However, since information on the passive transfer of immunity in canine visceral leishmaniasis (CVL) is lacking, serologically positive offspring may be being sacrificed when they are merely exhibiting passively-acquired antibodies. To evaluate passive transmission of humoral immunity in CVL, we used 10 serologically and parasitologically positive female dogs and their 48 puppies, in which IgG and IgM levels were evaluated using indirect immunofluorescence reaction over a period of four months. Passive transmission of cellular immunity was analyzed using the delayed test of hypersensitivity (DTH) in another group of 5 female dogs naturally infected and their 25 offspring that were between 8 and 75 days old, as well as a control dog and her 9 puppies 8 days after birth. IgG-class antibodies were identified in all the litters starting from the day of birth and lasting on average 32.1 ± 15.3 days (14-70 days). IgM was not detected in the offspring but was present in 7 of the 10 adult dogs. Among the adults with positive DTH, 72% of their puppies also had positive DTH reactions. Passive transmission of humoral and cellular immunity occur in CVL and therefore it is recommended that seropositive offspring less than 70 days of age should not be sacrificed based on serologic surveys for CVL.

KEY-WORDS : passive immunity - canine visceral leishmaniasis - Leishmania chagasi.

Introduction

Visceral leishmaniasis is becoming more frequent in many areas of the world due to epidemiological changes, like urbanization in Brazil and mass migrations on the Indian subcontinent, which lead to the introduction of non-immunized individuals into the disease cycle [27].

In many regions where the zoonotic visceral leishmaniasis (ZVL) is endemic, dogs are considered the principal reservoir. This occurs in countries in southern Europe, the Middle-East Asia and northern Africa where the etiologic agent is Leishmania infantum, as well as Central and South American countries where the disease is caused by Leishmania chagasi [5].

ZVL control is limited and includes combating the vector, eliminating reservoirs and treating the infected human population [8,17]. These measures can help limit ZVL transmission in endemic areas, as was shown by MAGALHÃES et al. (1980) [18] in Vale do Rio Doce, Minas Gerais, Brazil.
Canine visceral leishmaniasis (CVL) diagnosis can be made based on serologic tests. Once the antibody response is exceptionally strong [26], a serologic survey of the canine population is one of the measures that has been adopted to identify positive animals to be sacrificed as a form of disease control. However, because information on the transfer of passive immunity in CVL is lacking, serologically positive offspring may be sacrificed when in fact they are merely exhibiting passively-acquired antibodies.

The immune relationship between mother and neonate is complex. Antibodies, parasitic antigens, immune cells, cytokines and probably other still-unknown factors may be transferred during the fetal phase across placenta and/or postnatally [6]. In dogs, as in cats, the placenta is the endotheliocorial type, in which the epithelium of the chorion is in contact with maternal capillary endothelia. In these species, small quantities of immunoglobulin G (IgG) (5-10%) may be transferred from mother to fetus, but the majority is obtained through the colostrum [25].

The period of intestinal permeability of proteins in offspring varies between species and between isotypes. Generally it is higher immediately following birth and declines after about 6 hours, possibly because intestinal cells that absorb immunoglobulins are replaced by a population of more mature cells. The length of the absorption period is not well established [25].

Normally, non-nursing animals have extremely low serum levels of IgG. Immunoglobulins, particularly IgG, is coming to the serum through colostrum absorption. Due to the nature of the absorption process, IgG peak serum levels usually occur between 12 and 24 hours postpartum. These passively-acquired antibodies immediately begin to decline through normal catabolic processes once absorption ends. The rate of decline differs depending on the Ig isotype, however the time required to reach non-protective levels also depends on initial concentration [25].

Passively-acquired maternal antibodies do not only inhibit Ig synthesis, they also avoid successful vaccination of young animals. This refractory period can last several months, depending on the quantity of antibodies transferred to the neonate and the half-life of the immunoglobulins [25].

There is evidence that cell-mediated immunity is transferred to newborns through colostrum [25]. Parasite-specific skin tests were positive in children of mothers infected with *Schistosoma mansoni* [6] and cellular immunity, mediated by antigen-specific T cells, was passed through maternal milk in mice infected with *Trichinella spiralis* and that immunity may have been protected the offspring against intestinal pathogens [15].

The maternal-fetal exchange, mainly the passage of molecules from maternal to fetal circulation is well-established, but the quantification and the effects of this exchange, particularly in CVL, are not documented. Information is necessary regarding levels of passive immunity in newborn dogs, which is of significant importance considering that serologically positive animals are sacrificed in ZVL control campaigns. Such information can help prevent the unnecessary sacrifice of uninfected dogs that are seropositive due to the passive transfer of antibodies.

2. Material and Methods

A) ANIMALS

Sixteen adult female dogs with confirmed gestation were studied. Fifteen dogs were diagnosed with visceral leishmaniasis through specific anti-*Leishmania* antibody detection using indirect immunofluorescence test and parasitologic examination of Giemsa-stained bone marrow smears. An adult female that was negative on the same tests was used as a control. The positive dogs were naturally infected and were obtained from the kennel of the Zoonosis Control Center of the City of Belo Horizonte, Minas Gerais. The control dog came from our study kennel (Institute of Biological Sciences of the Universidade Federal de Minas Gerais). A total of 82 offspring were part of the study starting from the day of birth. The study animals were of the following breeds: Two adult Dobermans with 16 offspring; 1 adult Rotweiller and 8 offspring; 1 adult Boxer with 8 offspring and 12 adults of mongrels with 50 offspring. The adults, together with their litters, were maintained in separate cages. The animals were identified by number according to the order of entrance into the study. Offspring received the same number as their mother, followed by a letter in alphabetical order.

After weaning, puppies and adults were fed an age-appropriate, balanced diet (Purina® *ad libitum*). All animals were inspected daily and examined weekly. Periodic evaluation of the environment was done and pyrethroid insecticide (K-Othrine®) was applied quarterly to have a control the presence of sandflies.

B) PLASMA AND MILK COLLECTION

Blood and milk samples of 10 adult females and blood samples of their 48 offspring were collected on the day of birth and weekly for 120 days postpartum. Punctures were made in the jugular vein of puppies and the radial vein of adults to draw blood samples using disposable sterile syringes containing heparin. The plasma was separated, aliquoted in individual vials, marked and stored at -20°C until use.

C) OBTAINEMENT OF ANTIGENS FOR IMMUNOLOGIC TESTS

a) Antigen for indirect fluorescent antibody assay (IFA).

*L. mexicana* (strain MHOM/BR/1960/BH6) promastigotes were used, which had been maintained in logarithmic growth in liver infusion triptose culture medium. After 48 hours of cultivation, the parasites were fixed in 5% formaldehyde for thirty minutes followed by 500-g centrifugation for 10 minutes. The sediment was resuspended in PBS, pH 7.2 and
submitted to further centrifugation. This process was repeated three times and the sediment was then resuspended in PBS. Ten microliter of the suspension were placed on slides for immunofluorescence with a concentration of 10-20 promastigotes per field. After being air-dried, the slides were wrapped in blotting paper then aluminum foil and stored at -20°C until use.

b) Antigens for delayed hypersensitivity skin tests.

Antigens used for the skin test were made separately from extracts of two Leishmania strains: L. (L.) chagasi (strain MHOM/BR/1972/BH 46) and L. (V.) braziliensis (strain MCA3/BR/1972/C348) according to the method described by MELO et al. (1977) [22].

D) IMMUNOLOGIC TESTS

a) Humoral immunity.

Was evaluated by IFA according to the method described by CAMARGO (1964) [4] in the plasma and milk of adults and in the plasma of offspring using anti-IgG (Biomanguinhos, Fiocruz, Rio de Janeiro) and anti-IgM (Bethyl-Laboratories, Inc., USA). The results were expressed in antibody titer, which were obtained by serially diluting the plasma by a factor of 2 until a negative result was reached. IgG and IgM were analyzed in all samples collected from puppies starting on the day of birth until 120 days postpartum.

b) Cellular immunity.

Was analyzed by skin test to evaluate the delayed hypersensitivity type of response. The test was performed on the control animal and her litter (n=9) at 9 days postpartum and in 5 adult females and their respective litters that were aged 8, 15, 36, 39, 57 and 75 days.

Each animal received 200 µl of antigen containing 200 µg of protein via the intradermal route on the inside of the thigh according to the technique described in GENARO et al. (1988) [9]. The L. (L.) chagasi antigen was injected into the right thigh and the L. (V.) braziliensis antigen into the left, both solutions were prepared as described above. All adults were given the test on the same day as their litter. Animal 13 had a litter of 10 offspring, half of which received the skin test at 8 days of age and the other half at 75 days postpartum.

The diameter of the injection site was measured 72 hours after the test was given. Reactions measuring 5mm or more were considered positive [9].

Results

A) IgG TITER

IgG-class anti-Leishmania antibodies were detected in all litters. These antibodies were present in the plasma of offspring starting on the day of birth and in the blood, colostrum and milk of seropositive adults.

It was possible to collect blood from the offspring of adult number 4 (Figure 1) before they had contact with maternal colostrum. These samples did not have anti-Leishmania antibodies but the next samples (day 7) had an average titer of 1:220, which decreased until day 28, the last collection that contained antibodies.

Adults’ plasma IgG levels were constant in adults numbered 1, 4, 5, and 10. The remainder had slight fluctuations during the postpartum period. That was not the case with IgG in the milk of all adults, which declined in general between days 7 and 14.

Table I lists the plasma and milk antibody titers of adult dogs on the day they gave birth, the average IgG titer during the nursing phase and the duration of IgG in the milk and in the plasma of offspring. The duration of passive anti-Leishmania antibodies in the offspring varied between 14 and 70 days with an average of 32.1 ±15.3 days.

B) IgM TITER

Table I shows the results of IFA analysis of IgM. The adult females’ IgM titer on the day of delivery varied between 1:20 and 1:80, with 3 of the 10 tested adults presenting negative IFA for this immunoglobulin. None of the offspring had positive IgM on any of the tests, which were performed on samples collected on the day of birth and weekly until 120 days postpartum.

C) DELAYED TEST OF HYPERSENSITIVITY (DTH)

All leishmaniasis seropositive adults used in this study also had positive reactions on the DTH (skin test) for one or both of the Leishmania antigens studied here.

The results for each animal can be seen in Table II along with the animal’s age and the increase in size in millimeters at the application site of each antigen. Of the 25 offspring of positive mothers used in this experiment, 18 (72 %) had positive results. Of the seven with negative results, two were from the same litter and were 39 days old while the other 5 also came from one litter and were 75 days old. However, when only offspring less than 60 days old were considered, 18/20 (90 %) were positive, and in tests done after this age,
none of 5 that were originally positive were reactive. The control animal and her litter (n=9) were not reactive to any of the antigens.

The litter of animal 13 was tested at two different ages. Half the litter was tested at 8 days old, with 100% positive and the other half at 75 days old, with 100% negative (Table II).

Figure 2 illustrates the number of offspring tested and the number of positive reactions on the DTH according to age on the day of the test.

Discussion

VL diagnosis can easily be made based on serologic tests. Exceptionally strong reactions to VL antibodies indicate the presence of a large number of parasites in the bone marrow or the spleen, organs that play a fundamental role in the production and subsequent maturation of antibodies [26].

In Brazil, serologic survey of canine populations is one of the measures that has been adopted to identify positive animals to be eliminated as a means of controlling the disease. However, as there is not adequate data related to the passive transfer of immunity in CVL, serologically positive offspring may be being destroyed when they are merely exhibiting passively-acquired antibodies.

In dogs, passive antibody transmission is better described for viral diseases like canine distemper, parvoviruses and infectious canine hepatitis. An in-utero presence of canine distemper-neutralizing antibodies in gnotobiotic litters deprived of colostrum and in offspring free of pathogens has been observed. These maternally-derived antibodies protected the offspring from fatal infection with the virulent canine distemper virus. This protection was associated with lymphope-
nia, viremia and the temporary suppression of the lymphatic response to PHA-P [14]. In the presence of maternal antibodies, the ability of vaccines to elicit a serologic response is dose related (antibody titer) [3]. Newborn hamsters of mothers infected with *L. donovani* responded better to immunization with amastigotes than offspring of uninfected mothers. This suggests specific sensitivity, which could have been provoked by the passage of soluble parasite antigens or from the sensitized cells of the mother to fetus during gestation and/or nursing [12].

The leishmaniasis are chronic afflictions in which, as is expected, there is an important production of IgM in the initial stages of the disease and subsequently the development of a predominantly IgG response [16]. The present study has proven that IgG-class anti-*Leishmania* antibodies are transferred from positive mothers to their offspring, since this type of immunoglobulin was detected in all the litters in concentrations that were inversely proportional to the age of the offspring. Even though IgM was not detected in 0-120-day-old puppies, the diagnosis of acute infection in offspring through the detection of specific IgM cannot be recommended due to the fact that this immunoglobulin is not always be detected in the acute phase of CVL [7, 11, 19].

The present study showed that passive transfer of IgG-class antibodies occurs through colostrum since these antibodies were not detected in offspring that did not ingest the colostrum of their infected mothers, yet were identified shortly after the first nursing session. Passively-acquired antibodies remained between 14 and 70 days with average duration of 32.1 ±15.3 days. In dogs and cats, postpartum transmission of immunoglobulins is not limited to a few hours after birth but can continue for a long period, often extending over almost the entire course of lactation [2].

In all the litters of infected mothers studied here, antibody levels in the offspring declined quicker than in the milk, which can be explained by both the decrease in immunoglobulin permeability in the intestine [25], as well as by the young animals’ rapid growth since this growth increases the amount of blood and consequently dilutes the concentration of maternal antibodies [2].

These results are important in determining the best age to vaccinate animals against leishmaniasis, when such vaccine is available. According to TIZARD (1996) [25], maternal antibodies do not only inhibit immunoglobulin synthesis but they also avoid successful vaccination in young animals and the duration of these antibodies, called the refractory period, depends on the quantity of transferred antibodies and the half-life of the involved immunoglobulins.

On the other hand, it is important to determine the age at which serologically positive animals can be sacrificed without the risk of them being positive due to passive immunity. As seen here, IgG-class anti-*Leishmania* antibodies were detected in puppies up to 70 days postpartum, which indicates that canine offspring exhibiting positive serology for leishmaniasis up to that age should not be sacrificed.

A positive serologic test in dogs less than 90 days old could raise the suspicion of premature infection via phlebotomines shortly after birth. However, GENARO et al. (1988) [9] and MELO et al. (1998) [21] showed that in dogs, IgG and IgM anti-*Leishmania* antibodies were only detected between 3 and 5 months after experimental infection with *Leishmania (L.) chagasi*.

The evaluation of cellular immunologic response to leishmaniasis in dogs using DTH has been performed by several authors who used antigens of different *Leishmania* species [1, 9, 10, 13, 20, 23, 24]. Analyzing cellular response in offspring using the DTH and *L. chagasi* and *L. braziliensis* antigens, we found that *L. chagasi* antigens had good results for this type of test and that 90% of offspring under 60 days of age were reactive to *L. chagasi* and/or *L. braziliensis* anti-

<table>
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<th>Adult/offspring (number)</th>
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*Results from test done when offspring were 8 days old.

Table II. — Results of delayed test of hypersensitivity using *Leishmania* antigens in adult female dogs with visceral leishmaniasis. Adults are represented by number and their respective offspring are identified by that number followed by a letter.
gens, whereas not one of the puppies older than 60 days had this type of reaction.

These data regarding passive transmission of cellular immunity seem to indicate that mothers with positive DTH for *Leishmania* antigens have offspring with the same response, while this reaction is absent in animals over 75 days old. These results agree with those of TIZARD (1996) [25], which hold that there is evidence that cell-mediated immunity can be transferred to newborns via colostrum.

Our data indicate that offspring of VL-positive mothers passively acquired cellular and humoral immunity and that immunity was transitory. Further experiments evaluating IgG subclasses and the phenotype of mononuclear cells could provide new insight to better understand the maternal-fetal relationship in CVL.

Based on these findings, we suggest that seropositive offspring, under 70 days of age, of infected mothers should not be sacrificed as part of ZVL control programs. Rather, these animals should be monitored in order to confirm diagnosis. This is an important measure considering the high psychological and/or commercial value of the animals, as well as the need to maintain genetic characteristics of certain races.

Acknowledgements

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References