Experimental cutaneous leishmaniasis IV. The humoral response of Cebus apella (Primates: Cebidae) to infections of Leishmania (Leishmania) amazonensis, L. (Viannia) lainsoni and L. (V.) braziliensis using the direct agglutination test

Lourdes Maria Garcez a,*, Fernando Tobias Silveira a, Abdallah El Harith b, Ralph Lainson a, Jeffrey Jon Shaw a,c

a Leishmaniasis Program, Evandro Chagas Institute, Caixa Postal 3, 66017-970, Belém, Pará, Brazil
b Afdeling Medische Microbiologie, Universiteit van Amsterdam, Amsterdam, Netherlands
c Parasitology Department, Institute of Biomedical Sciences, São Paulo University, São Paulo, Brazil

Received 18 November 1996; received in revised form 16 April 1997; accepted 23 April 1997

Abstract

The direct agglutination test (DAT) was used to evaluate the serological response of 150 serum samples taken from 15 captive-bred capuchin monkeys Cebus apella. These animals had been experimentally infected with either L. (Leishmania) amazonensis, L. (Viannia) lainsoni or L. (V.) braziliensis. Monkeys infected with L. (L.) amazonensis or L. (V.) lainsoni were challenged with the homologous parasite one month after their spontaneous cure. DAT antigens were prepared from L. (L.) donovani, L. (L.) amazonensis and L. (V.) braziliensis. Antigens were difficult to standardise and it was impossible to produce an L. (V.) lainsoni antigen as parasites remained aggregated even after trypsinization. The DAT detected significant humoral responses in all the infected monkeys. Titres were higher when homologous antigens were used, especially in secondary responses. This suggests that homologous antigen should be used to detect antibodies in human cutaneous leishmaniasis. © 1997 Elsevier Science B.V.
Keywords: DAT; Cebus apella; Experimental cutaneous leishmaniasis; L. (Leishmania) amazonensis; L. (Viannia) braziliensis; L. (V.) lainsoni

1. Introduction

Cutaneous leishmaniasis is a zoonosis caused by parasites of the genus Leishmania and is transmitted amongst silvatic animal reservoirs by sandflies (Psychodidae: Phlebotominae). Man and domestic animals are accidental hosts and in patent infections a primary lesion develops at the site of the insect bite. Progression of the disease varies and there may be spontaneous cure or an evolution to grave pathologies (disseminated, mucosal or diffuse leishmaniasis) that in some cases are mutilating and even incurable.

In the New World deforestation, forest management or any activity that involves an intimate contact with the forest ecosystem can result in outbreaks of cutaneous leishmaniasis in man. In the Amazon region different species of Leishmania coexist and each has its own epidemiology and transmission cycle. An important aspect of the epidemiology of cutaneous leishmaniasis in this region is that a person may be infected more than once. The reasons for this are not fully understood but it is possible that different parasites do not protect against others and that the course of immunity also varies with different species. Since both reservoirs and vectors are silvatic, the only feasible control against cutaneous leishmaniasis is by vaccination. However, so far, no suitable vaccine for wide scale use in the Amazon region has been developed.

In the development of a vaccine it is necessary to study the modified immunological course that candidate antigens produce in animal models. The immune response associated with T-lymphocytes would seem to be the prime modulator of leishmanial immunity (Scott et al., 1989; Sadick et al., 1986; Coffman et al., 1991), however, activated T-cells also interact with B-cells to produce specific antibody (Alberts et al., 1994). A number of factors effect the choice of the animal species and amongst these are the genetic distance from man and the course of the infection.

The monkey Cebus apella has been successfully used for experimental studies of American cutaneous leishmaniasis. Phylogenetically this animal is closer to man than rodents and the course of primary response of experimental leishmanial infections is very similar to that observed in man (Lainson and Shaw, 1977; Silveira et al., 1989, 1990a,b).

The DAT has been shown to be both a sensitive and specific test for the serodiagnosis of visceral leishmaniasis in man (Harith et al., 1986, 1988, 1989; Andrade et al., 1987, 1988, 1989). Antibodies were also detected in cases of human cutaneous leishmaniasis with the DAT (Mengistu et al., 1990) in Ethiopia. In these studies the antibody titres were similar using DAT antigens prepared from four different Old World Leishmania species. However, the considerable taxonomic and clinical differences between parasites of the two subgenera that occur in the
Americas prompted us to examine the antigenic specificity of DAT antigens in greater detail.

One of the great advantages of the DAT is that unlike most assays used to detect antibodies it does not require a species specific anti-immunoglobulin conjugate. Thus it has the potential of detecting leishmanial antibodies in both wild reservoirs and in experimental infections of animals for which conjugates are not available.

In the present study the DAT is used to follow the humoral response of C. apella experimentally infected with Leishmania (Leishmania) amazonensis, L. (Viannia) lainsoni and L. (V.) braziliensis, using both homologous and heterologous antigens.

2. Materials and methods

2.1. Monkeys and sera samples

Fifteen young C. apella monkeys, born in captivity, were used in this study. They were divided into three equal groups that were each inoculated with one of the following parasites: I, L. (L.) amazonensis; II, L. (V.) lainsoni; and III, L. (V.) braziliensis. The animals of groups I and II were reinoculated 1 month after the spontaneous cure of the first infection, which lasted for 4 months. Group III animals were not reinoculated because their initial infections lasted for periods of up 5–10 months. The animals were bled before inoculation, 15 days post-inoculation and then regularly every 30 days. The course of the cutaneous lesions in all groups has been previously described (Silveira et al., 1989, 1990a,b). A total of 150 sera samples were tested by the DAT.

2.2. Leishmania species and culture

Promastigotes of L. (L.) donoani, L. (L.) amazonensis and L. (V.) braziliensis (strains MHOM/IN/88/DD8, IFLA/BR/67/PH8 and MHOM/BR/75/M2904, respectively), were cultured in RPMI medium containing 10% foetal calf serum (FCS) and 1% GPPS. The stock solution contained glutamine 0.2 M, pyruvate 0.125 M, penicillin/streptomycin and 2-ME 50 mM. Each strain was harvested when the majority of the promastigotes were elongated according to a previously established growth curve.

2.3. Antigens

Promastigotes were collected (4000 × g at 4°C for 15 min) and the pellet was washed five times by centrifugation and resuspension (3200 × g at 4°C for 10 min) in cold Locke's solution that was kept at 4°C. After washing, a 0.4% trypsin solution was made in Locke's, the pellet was weighed (wet weight) and a volume of trypsin solution equal to 19 times that of the pellet was added. The organisms were incubated at 37°C for 45 min. After this they were washed again (five times) as described before and resuspended in cold Locke's solution at a concentration of
2×10^8 parasites/ml. An equal volume of 2% formaldehyde in Locke's solution was added to give a final concentration of 10^8 fixed parasites/ml.

Following fixation overnight (about 16 h), the parasites were washed twice by centrifugation (3200×g at 4°C for 10 min) in cold physiological saline, about 4°C. They were then stained with 0.02% (w/v) Coomassie Brilliant Blue, diluted in physiological saline, for 90 min, using a magnetic stirrer at a moderate speed. The stained promastigotes were washed twice with physiological saline, as described before and, finally, resuspended at a concentration of 10^8 parasites/ml in physiological saline containing 1% formalin. This antigen was stored at 4°C until used (Harith et al., 1986).

2.4. Test procedure

C. apella sera were treated with 2-mercaptoethanol 0.2 M by addition of 70 μl to 10 ml of sera diluent (physiological saline with FCS 1%). Serial two fold dilutions of sera were prepared from 1/20–1/5120. After dilution they were incubated at 37°C for 1 h. An equal volume of antigen (50 μl) was then added to each well (‘V’ shaped wells) and completed plates were kept for 16 h (overnight) at room temperature. The reading was visual and the end-point estimated as being the well in which there was a clear sharp-edged blue spot identical to the one observed in the control well that contained saline/antigen (Harith et al., 1986, 1988, 1989).

3. Results

3.1. Leishmania growth curves in RPM I

Species of the subgenus Leishmania grew satisfactorily in RPM I medium when a seed culture containing from 4 or 5×10^5 parasites/ml was used. Rocking improved the growth but it was not essential for good development. Larger inocula (10^6 parasites/ml) produce greater numbers of organisms (Figs. 1–3).

3.2. Antigen standardization

Initially we had difficulties in producing the antigen—some lots worked well whereas others did not. The major difficulty was that it was impossible to distinguish positives and negatives. Furthermore, with some lots the negative button had a very irregular form, making it impossible to interpret the results. After many attempts, the following factors were identified as being crucial:

- the method of preparation must be followed exactly every time;
- parasites must be cultured in liquid medium using preferentially a rocker platform to provide a greater number of flagellates;
- the water used to make all the solutions must be distilled and deionised;
L. M. Garcez et al. / Acta Tropica 68 (1997) 65–76

Fig. 1. L. (L.) donovani growth curve in RPMI medium. The mean number of parasites per milliliter in five culture flasks has been estimated on each day. Inocula, $0.4 \times 10^6$/ml (5 ml/flask).

- promastigotes must be washed ten times in Locke's solution and during all washes the Locke's solution must to be cold, about 4°C, otherwise the antigen will not work. It is also advisable to use cold physiological saline for the other washes;
- during trypsinization the parasites must be incubated at about 37°C for 45 min. Incubating for longer periods or at other temperatures could kill many of the organisms. It is essential that all the promastigotes are alive when fixed;
- a strong indication that the antigen will work is its colour which should be a light blue tone.

It proved impossible to produce a L. (V.) lainsoni agglutinating antigen. The growth of this species, described by Silveira et al. (1989), in culture is characterised by large aggregations that can be up to 2 mm in diameter. These are composed of parasites attached to each other by their flagella and trypsinization (0.4% trypsin at 37°C for 45 min) failed to free them.

Fig. 2. L. (V.) braziliensis growth curve in RPMI medium. The mean number of parasites per milliliter has been estimated on each day. Inocula, $0.5 \times 10^6$/ml (5 ml flask).
3.3. Effect of 2-mercaptoethanol

Other workers have reported that the specificity of the DAT can be improved by the use of 2-ME (Harith et al., 1986, 1988; Peralta et al., 1981). However, when eight sera from non-infected monkeys were diluted with 2-ME there were no significant differences in their titers (Table 1).

3.4. Cebus apella humoral response

Each group of monkeys had a distinct humoral response, which depended on the species of parasite inoculated and the species used to prepare the DAT antigen.

Table 1
A comparison of the effect of 2-ME on the sera of eight negative control Cebus apella using an antigen of Leishmania (Leishmania) donovani in the direct agglutination test

<table>
<thead>
<tr>
<th>Sera</th>
<th>Titres of the direct agglutination test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without 2-M E</td>
</tr>
<tr>
<td>1</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>640</td>
</tr>
<tr>
<td>3</td>
<td>320</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>320</td>
</tr>
<tr>
<td>8</td>
<td>160</td>
</tr>
</tbody>
</table>

a Seventy milliliters of 2-M E 0.2 M + 10 ml of saline with FCS 10% (~14 mM of 2-M E).
Fig. 4. Mean DAT* antibody titres using three antigens of five Cebus apella (Primates: Cebidae) inoculated with Leishmania (L.) amazonensis. * Direct agglutination test; ** first agglutination; *** second agglutination.

3.4.1. C. apella inoculated with L. (L.) amazonensis (Fig. 4)

There were three different levels of secondary humoral response that were related to the different DAT antigens. The primary response was similar and very low with all three antigens. However, it is evident that the antibody response stimulated by the homologous challenge was higher when measured using the homologous antigen. The highest DAT titres with all three antigens always occurred during the first month after the second inoculation of Leishmania. The titres were higher with the L. (L.) amazonensis homologous antigen but were detectable at lower levels with the two heterologous antigens of L. (L.) donovani and L. (V.) braziliensis.

3.4.1.1. Course of infection. The development of L. (L.) amazonensis lesions was similar in all five monkeys except the incubation period. The mean number of lesions per monkey was 5.7. In four monkeys, the lesions appeared approximately 20 days post inoculation (p.i.) as small erythematous papules that by 2 months p.i., had developed into nodules. By 3 months p.i., all the lesions had healed except for those of one animal in which the lesions only appeared 2 months p.i. and remained for 4 months p.i. All five monkeys were cured by 5 months p.i. and at this time they were immune to an infective homologous parasite challenge.

3.4.2. C. apella inoculated with L. (V.) lainsoni (Fig. 5)

The profile of the humoral response of these animals was different from those that had been inoculated with L. (L.) amazonensis. In this group the humoral response was only detectable when the L. (V.) braziliensis antigen was used and it continued to increase after the secondary challenge of live parasites. The primary response using this antigen was higher than that observed in the monkeys infected with L. (L.) amazonensis. Furthermore, the titres began to rise 2 months after the
3.4.2.1. Course of infection. L. (Viannia) lainsoni infections were similar to those of L. (L.) amazonensis and like the latter the lesions did not ulcerate. Although the mean number of lesions was slightly higher (6.6) the lesions remained active for a longer period. After one month p.i., all of the animals had erythematous papules that developed into small nodules at 2 months p.i. After 3 months p.i., four animals still had lesions (erythema and infiltration) and one was cured. Nevertheless, parasites were only found in the stained smears of one of the monkeys that had lesions. Four months after inoculation the lesions had regressed in all the animals. All the animals of this group were immune to homologous challenge with infective parasite.

3.4.3. C. apella inoculated with L. (V.) braziliensis (Fig. 6)

The antibody response of this group was only related to the primary challenge as they did not receive a secondary challenge. The antibody titres were, however, higher than those of the other groups during the first 3 months, especially with the homologous antigen but they did not reach the antibody levels stimulated by the secondary challenge with the homologous antigen.

3.4.3.1. Course of infection. The infection in the group of monkeys inoculated with L. (V.) braziliensis was much longer than those of the other two Leishmania species. Erythematous lesions appeared in 5 animals between 15–20 days p.i. One month
after inoculation the lesions were erythematous papules that between 1–2 months (mean number of lesions equal to 7 per animal) underwent spontaneous ulceration. Three months p.i., the lesions showed signs of cicatrization, but parasites could still be found up to 6 months p.i. In this group the clinical cure took place from 4–8 months p.i. These monkeys were not immune to the homologous challenge.

4. Discussion

To ensure that batches of high quality antigen can be repeatedly produced it is absolutely essential that the protocol is followed in every detail. It was remarkable how just small variations in the protocol, which at the time were thought to be unimportant, deleteriously affected the antigen to such a point that it was unusable. It is also essential that the parasites are grown in liquid medium because very large numbers of organisms are required. For instance 10 billion parasites are needed to produce 100 ml of DAT antigen. Furthermore, it may be impossible to prepare a DAT antigen from certain species of Leishmania, such as L. (V.) lainsoni, because they grow in clumps which cannot be broken up by trypsinization.

The DAT successfully detected antibodies in the sera of C. apella that had been experimentally infected with three different Leishmania species. Silveira et al. (1989, 1990a) described the experimental infections in C. apella of L. (L.) amazonensis, L. (V.) lainsoni and L. (V.) braziliensis. Later they evaluated the importance of this monkey as a model for studying cutaneous leishmaniasis and concluded that the pathologies of the lesions produced by the different parasites were similar to those seen in patients (Silveira et al., 1990b). The present study
shows that agglutinating antibody levels of experimentally infected C. apella were usually low in the primary response and that they were more evident in monkeys inoculated with L. (V.) lainsoni and L. (V.) braziliensis. Nevertheless, the successful detection of antibody antigen depended on the source of antigen used in the DAT. Lujan et al., 1987 detected low IgM levels during the primary and secondary experimental infections of L. (V.) panamensis in Aotus triirrgatus, using a homologous antigen in an ELISA. In these same animals leishmanial IgG antibody production was boosted by the challenge, reaching titres of 5120.

The present study indicates that the use of a homologous antigen for the DAT is more important when measuring antibodies produced by the second infection. However, in the case of L. (V.) lainsoni infections the primary antibody response was only detectable with the subgeneric homologous L. (V.) braziliensis antigen. In these same animals neither the primary nor secondary response was detectable using the two antigens made from parasites belonging to the subgenus Leishmania. It would appear that the higher titres that resulted from the second inoculation of living parasites in C. apella are a reflection of a humoral response to species, species complex or group specific Leishmania antigens. This is emphasised by the fact that the secondary response of the monkeys infected with L. (L.) amazonensis was higher with the homologous antigen than with the subgeneric homologous L. (L.) donoani antigen. Mengistu et al. (1990) reported that antibody titres were similar in cases of Ethiopian cutaneous leishmaniasis when the DAT was performed with L. (L.) aethiopica, L. (L.) major, L. (L.) tropica and L. (L.) donoani antigens. However, these authors observed that the L. (L.) tropica antigen reacted non-specifically at lower levels with sera from patients with discoid lupus erythematosus and malaria.

In epidemiological surveys it is generally considered that intradermal reactions rather than serology are a more useful measure of cutaneous leishmaniasis endemicity. Serology in these cases relates to ELISA and IFA tests. In endemic areas patients are being continuously challenged with parasites because they are frequently bitten by infected sandflies. Because of their immunity, however, they may not develop lesions. This is similar to the L. (L.) amazonensis model in which challenge resulted in a pronounced specific humoral response but no lesion developed. One might expect that in such cases their humoral response will parallel the secondary response seen in our monkey model rather than the primary one. It remains to be seen therefore, how sera from patients from endemic regions react with DAT antigens made from the different parasites that are known to occur in man in these same areas.

With the most homologous antigen the DAT antibody titres clearly reflected a humoral response of C. apella to experimental cutaneous infections with the three different parasites. In this respect it is interesting to note that the IFAT results (unpublished observations) of the same monkeys used in the present study were inconclusive and titres were low and did not relate to the phase of the infection as did the DAT results.
5. Conclusions

The DAT is particularly useful because it does not require a species specific immunoglobulin conjugate for the evaluation of a host's humoral response to infection with *Leishmania* as do other tests such as the ELISA and IFAT. It may also be measuring antibodies that are not detected by the other methods. Individual differences in *C. apella* humoral response could be observed with DAT, showing its importance in longitudinal studies. The test was efficient in demonstrating the humoral response of *C. apella* to experimental cutaneous infections with *L. (L.) amazonensis*, *L. (V.) lainsoni* and *L. (V.) braziliensis*. Titres were always higher with homologous antigens, specially in the secondary responses. This suggests that homologous antigens should also be preferentially used for detection of antibodies in cases of human cutaneous leishmaniasis.

Acknowledgements

We would like to thank Dr Paulo Paes de Andrade for being always open for discussions and Dr José Augusto P.C. M uniz, the Director of the National Primate Center, Belem-Pará, Brazil, for providing laboratory bred specific pathogen free monkeys. This investigation received financial support from the Evandro Chagas Institute.

References


Lujan, R., Hanson, W. L., Chapman, W. L., Dennis, V. A., 1987. Antibody responses, as measured by the enzyme-linked immunosorbent assay (ELISA), in owl monkeys experimentally infected with Leishmania braziliensis panamensis. J. Parasitol. 73, 430–432.


