A rapid and sensitive method for the identification of *Leishmania* with monoclonal antibodies using fluorescein-labelled avidin

J. J. Shaw, E. A. Y. Ishikawa and R. Lainson

When identifying *Leishmania* with monoclonal antibodies (Mabs) either radioimmunoassay (RIA) or indirect fluorescent antibody (IFA) tests have been used (GRIMALDI et al., 1987; SHAW et al., 1987). The RIA test is very sensitive, but because it uses radioactively labelled antibody it is not suitable for many developing countries. The IFA test does not use radioactive substances and is therefore more useful in such countries, but it has the disadvantage of being less sensitive than the RIA. In looking for a more sensitive non-radioactive method to test anti-leishmanial Mabs we tried different methods that use avidin/biotin systems. These methods are gaining popularity because the strong avidin/biotin binding increases sensitivity, and means that smaller quantities of primary serum are required (CROCKER & KUHN, 1983; CONDON et al., 1988).

Tests were performed with logarithmic serial dilutions of the following 12 anti-leishmanial mouse Mabs (primary sera): B2, B5, B12, B13, B18, B19, D13, L1, M11, M12, T3 (from Dr D. McMahon-Pratt, Yale University, USA), WIC 79.3 from Dr D. Snary, Wellcome Foundation, UK). We used commercially produced biotin and avidin reagents (Vectastain®Vector Laboratories, Burlingame, California, USA) and culture forms of the following *Leishmania* species: L. (Leishmania) amazonensis (IFLA/BR/68/PH8), L. (L.) major (PSAM/IL/83/PSAM398), L. (Viannia) braziliensis (MHOM/BR/75/M2903), L. (V.) guyanensis (MHOM/BR/75/M4147), L. (V. ) naiffi (MDAS/BR/79/M5533). Promastigotes were washed 3 times in phosphate-buffered saline (PBS), distributed on multi-spot slides, air-dried, fixed for 10 min in acetone, and stored in sealed plastic bags containing dry silica gel at -70°C until used.

Slides were first incubated in PBS containing normal horse serum (1·5–3%) to block possible non-specific reactions caused by the secondary serum, biotinylated anti-mouse immunoglobulin (Ig) G (Vector BA-2000), which is prepared in horses. After incubation with the Mabs and biotinylated anti-mouse IgG we used (i) Vectastain® ABC (peroxidase) (Vector PK-4000), (ii) Vectastain® ABC-phycocerythrin (Vector EK-7000) or (iii) fluorescein avidin D (Vector A-2000). Slides were examined microscopically with a ×40 objective using light of the correct wavelength for each product. The stability of the Mabs was improved by dilution in PBS (pH 7·2) containing 0·1% bovine serum albumin and 15% glycerol. The fluorescein avidin D was titrated in 10 mm HEPES buffer in 0·15 mm NaCl containing 0·08% sodium azide. The most suitable working dilution was between 1/80 and 1/160. All serial logarithmic dilutions of the primary serum (mouse Mab) were also tested against the same promastigote antigens in an IFA test using an anti-mouse Ig conjugate and the method described by SHAW et al. (1987).

The test procedures for the two avidin/biotin complex products were those recommended by the manufacturer. The procedure for the indirect fluorescein-labelled avidin D (IFAV) test is given in the Table.

Table. The indirect immunofluorescent test using fluorochrome-conjugated avidin D

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
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<tbody>
<tr>
<td>1.</td>
<td>Incubate 15 min in normal horse serum.</td>
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<tr>
<td>2.</td>
<td>Blot off excess serum.</td>
</tr>
<tr>
<td>3.</td>
<td>Incubate 15 min in leishmanial monoclonal antibody (primary antibody).</td>
</tr>
<tr>
<td>5.</td>
<td>Incubate 15 min in biotinylated anti-mouse immunoglobulin (secondary antibody).</td>
</tr>
<tr>
<td>6.</td>
<td>Wash rapidly in running PBS.</td>
</tr>
<tr>
<td>7.</td>
<td>Incubate 15 min in fluorescein-labelled avidin D.</td>
</tr>
<tr>
<td>8.</td>
<td>Wash rapidly in running PBS.</td>
</tr>
<tr>
<td>9.</td>
<td>Mount in alkaline buffer (pH 9·5) containing 30% glycerol.</td>
</tr>
</tbody>
</table>

*If non-specific fluorescence is not a problem, steps 1 and 2 may be omitted or normal horse serum may be added at a concentration of 1·5–3% to the buffer used to dilute the monoclonal antibody.*

Non-specific reactions were noted with some Mabs when ABC-peroxidase was used. The level of fluorescence was disappointing low and short-lived with R-phycocerythrin. Fluorescein avidin D gave the best results with strong, stable specific fluorescence and very low background fluorescence. The maximum working dilution of the primary antibody (Mab) in the IFAV test was generally 1:1000 compared to only 1:100 in the standard IFA test. We recommend the IFAV test because it can be performed quickly, there are no non-specific reactions, and it is more sensitive than the standard IFA test which used a fluorescein-labelled secondary antibody.

Acknowledgements

This work was supported by Grant 17331 from the Wellcome Trust, and by the Fundação de Serviços de Saúde Pública of the Brazilian Ministry of Health. E.A.Y. is supported by a Brazilian Research Council (CNPq, Process 820348268) research fellowship. We are grateful to José Paulo Cruz and Raimundo Mendonça for technical assistance.

References

Concentration of Pentostam® in human breast milk

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The leishmaniases, caused by the parasitic protozoan Leishmania, constitute one of the six tropical diseases selected by the World Health Organization, are endemic also in many temperate countries, and can be acquired by travellers. The only drug available for the present with an acknowledged favourable therapeutic index is pentavalent antimony (SbV) complexed to derivatives of either gluconic acid (Pentostam®; Burroughs-Wellcome) or meglumine (Glucantime®; Rhone-Poulenc) (BERMAN, 1988).

A recent clinical experience, in which a lactating mother with cutaneous leishmaniasis was treated with antimony, raised the question of partition of antimony from the mother’s serum to breast milk and of potential toxicity to the baby. Since, in endemic regions, there may be an appreciable number of lactating mothers who are infected with Leishmania, we determined antimony levels in the mother’s serum and milk.

Case report

A 27 years old Sudanese woman weighing 69 kg presented to the National Institutes of Health Clinical Center with multiple skin ulcers. She had lived in the USA from 1984 to March 1986, visited Khartoum from April 1986 to September 1986, and then returned to the USA. In October 1986 she noticed painful nodules on both arms, which gradually increased in size and ulcerated by November 1986. When examined in February 1987, each arm bore crustated ulcers, 1·5 × 2 cm to 2·5 × 3 cm in size, with raised margins. Subcutaneous nodules were present on the left arm. Leishmania were cultured from a biopsy of one of the ulcers. The patient had a 2 months old baby and was lactating.

The patient was treated intravenously with Pentostam®. Other than on the first day, when 1000 mg Sb was administered, the Pentostam regimen used was 1400 mg Sb (approximately 20 mg Sb/kg) each day for 14 d. The lesions and nodules became smaller and the margins became less raised during therapy. The patient was instructed to express her milk and bottle-feed the infant during Pentostam therapy, so that potential toxicity to the baby would be avoided.

Laboratory investigations

Material serum and milk levels of antimony were determined before and after the fifth and fourteenth doses of 1400 mg Sb (20 mg Sb/kg). Total Sb (SbIII + SbV) was measured by plasma emission spectroscopy at 259-805 nm using a Beckman Spectrascan V sequential spectrophotometer (Spectrometers Inc., Andover, Massachusetts, USA). Data points depict Sb concentrations for two determinations at each time; curves fitted by eye join the mean values.

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Laboratory investigations

Material serum and milk levels of antimony were determined before and after the fifth and fourteenth doses of 1400 mg Sb (Figure). Serum Sb levels rose from a pre-treatment mean of 0·1 μg Sb/ml to a mean of 85 μg Sb/ml, 15 min after intravenous dosing. Serum Sb levels declined from their peak value with an initial elimination half-life of about 2 h. Peak serum Sb levels after administration of currently used regimens (20 mg Sb/kg) have apparently not been reported. The initial half-life and the trough level of antimony found by us were similar to values previously reported by REES et al. (1980) and CHULAY et al. (1988) after administration of about 10 mg Sb/kg.

Breast milk concentrations before dosing (mean of 0·7 μg Sb/ml) were higher than serum levels. After dosing, peak milk levels were achieved at 4 h, and

Figure. Serum and milk concentrations of antimony (Sb). Serum and milk were obtained from the patient at the indicated hours before and after the fifth and fourteenth doses of 1400 mg Sb (20 mg Sb/kg). Total Sb (SbIII + SbV) was measured by plasma emission spectroscopy at 259-805 nm using a Beckman Spectrascan V sequential spectrophotometer (Spectrometers Inc., Andover, Massachusetts, USA). Data points depict Sb concentrations for two determinations at each time; curves fitted by eye join the mean values.

Informal consent was obtained from the patient and she was treated according to the guidelines of the US HHS.