The immunology and serology of leishmaniasis’

IV. Results of Ouchterlony double diffusion tests

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CHAFFEE (1963) reported briefly that differing immuno-diffusion precipitation patterns could be obtained by using antigens prepared from Leishmania donovani, L. tropica and L. braziliensis diffused against antisera in the Ouchterlony double diffusion technique. He stated that antigen-antibody reactions in gel diffusion indicated that some strains of so-called L. braziliensis from the Americas were actually L. tropica, and another strain of so-called L. braziliensis was similar to L. donovani. We decided to investigate these precipitation reactions to ascertain if such similarities were meaningful or if common antigens were so numerous in all strains that precipitation reactions were not helpful.

MATERIALS AND METHODS

Most of the strains of Leishmania spp. used have been listed before (BRAY and LAINSON, 1965). They are:

- L.1. L. braziliensis, Brazil;
- L.2. L. donovani, Kenya;
- L.6. L. tropica, Israel;
- L.7. L. braziliensis, (s.l.), Panama;
- L.11. L. mexicana, British Honduras;
- L.13. L. donovani, India;
- L.15. L. braziliensis, (s.l.) “diffusa” Brazil;

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L.27. Leishmania sp. from the tuberculoid leprosy type of disseminated cutaneous leishmaniasis in Ethiopia (SCHALLER and SERIE, 1963)

**Antigens**

Antigen was prepared by growing leptomaniads in dried-agar-coated Thompson flasks on 190ml of modified NNN rabbit-blood agar with a fluid phase of 50ml of Hanks’s balanced salt solution. Leptomaniads were harvested after 6-10 days of growth, washed four times in phosphate buffered saline (PBS, pH 7.2) and then resuspended in twice their packed volume of distilled water. This suspension was then freeze-dried, ground and extracted twice in 20 times its volume of distilled water at 4°C for 24 hours. The suspension was spun and the supernatant freeze-dried. The dry soluble antigen was kept at -20°C. Usually leptomaniads from 12 Thompson flasks were collected to yield about 3g of soluble antigen. An antigen was also prepared by collecting the fluid phase of the medium, free of leptomaniads after growth of *L. mexicana* L.11 leptomaniads, and dialysing this fluid against distilled water for three weeks at 4°C. The fluid was then freeze-dried and used as antigen. Antigen for the diffusion tests was used as a 1/10 solution in PBS.

**Sera**

Many of the sera used have been described previously (BRAY and LAINSON, 1965). They were collected from patients with active or cured leishmaniasis, largely in South and Central America, or from infected monkeys in the laboratory (LAINSON and BRAY, 1966). Serum from man with tuberculosis and from animals immune to *Trypanosoma cruzi* and *T. rhodesiense* were also used. Rabbit antisera to the various strain antigens were prepared as follows:

Day 0: 10mg of antigen in 0.5ml of PBS, emulsified with 0.5ml of Freund’s incomplete adjuvant, was inoculated into the foot-pad.

Day 7: 10mg of antigen in 1ml of PBS was inoculated intravenously.

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1 Kindly supplied by drs. H. Fromentin and C. Serie.
Day 14: As for day 0.
Day 21: As for day 7.
Day 28: Rabbits were bled and serum was separated.
All sera were inactivated at 56°C for 45 minutes and stored with 1/5000 sodium azide at -20°C.

Double diffusion plates

2ml 2% Noble agar in PBS with 1/5000 sodium azide was employed on 3 x 1 inch microscope slides. A centre hole and six or eight surrounding holes were punched with cutters. The centre hole was commonly used to hold antigen and the surrounding holes were filled with undiluted serum. The reaction was allowed to proceed for three days at room temperature. The plates were washed in PBS for two to three days, in distilled water overnight and then stained for five minutes in 0.5% amido-black in 70% methanol, 20% distilled water, 10% acetic acid glacial. The plates were then washed in the methanol-water-acetic-acid mixture until the gel was nearly colourless but the precipitation arcs remained blue.

RESULTS

Table 1 shows the number of precipitation arcs obtained by diffusing the various antigens against rabbit antisera to the antigens. Table 2 shows the number of arcs obtained by using the various sera from infected or recovered patients and monkeys.

Table 1 – The number of precipitation arcs formed between leishmanial antigens and rabbit antisera

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<thead>
<tr>
<th>Antigen</th>
<th>L1</th>
<th>L2</th>
<th>L6</th>
<th>L7</th>
<th>L11</th>
<th>L13</th>
<th>L15</th>
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<td>L1</td>
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<td>L11 fluid phase</td>
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Table 2 – The number of precipitation arcs formed between leishmanial antigens and sera from patients or animals with, or recovered from, leishmaniasis

<table>
<thead>
<tr>
<th></th>
<th>Sera from man with</th>
<th>Sera from monkeys with</th>
<th>Mouse</th>
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<td>KA Brazil</td>
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<td>L11 Fluid phase</td>
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1Kala-azar.
2Espundia.
3Multiple cutaneous lesions.
4Costa Rica.
Sera from the following human patients gave negative results:

4 oriental sore, middle East;
1 chiclero’s ulcer, British Honduras;
1 single sore, Panama;
1 single sore, Peru;
3 “diffusa” leproid type, Ethiopia;
1 uta, Peru;
5 non-infected controls;
1 tuberculosis.

Other sera giving negative results were from:

4 monkeys recovered from L1 L. braziliensis;
6 monkeys recovered from L11 L. mexicana;
2 monkeys recovered from L7 L. braziliensis (s.l.), Panama;
1 rat recovered from T. rhodesiense;
5 monkeys recovered from malaria infection;
4 uninfected monkeys;
1 horse immunized with T. cruzi.

Two sera from patients with L. braziliensis “diffusa” type, one from Brazil and one from Venezuela, gave a single arc with some antigens. Both these patients, however, had received treatment before their sera were taken.

All kala-azar sera gave one or more arcs with all antigens. Disseminated cutaneous leishmaniasis, either as developed naso-pharyngeal involvement or multiple skin lesions, gave one or more arcs with nearly all antigens. No sera from patients or animals with a single cutaneous lesion gave a positive result. The monkey inoculated with L15 L. braziliensis (s.l.) “diffusa” type was positive to the leishmanin skin test and had been inoculated in four sites, giving rise to three major ulcerating lesions.

Several points of interest arise on study of the number and extent of the precipitation arcs. L. braziliensis (s.l.) antigen, of
a strain isolated from a case of leishmaniasis tegumentaria diffusa, shows a large number of arcs of similar extent in common with known L. braziliensis from Brazil. L. braziliensis (s.l.) from Panama stands on its own with no pronounced relationship to L. braziliensis from South America and even less relationship to L. tropica or L. mexicana (to the extent of no reaction between L. mexicana antigen and sera from costa-rican leishmaniasis). L. mexicana and L. tropica show little similarity, having only one arc in common. The two L. donovani strains do not display any great similarity, sharing only three arcs of a possible five, and they both share several common antigen-antibody complexes with L. tropica and L. braziliensis. The Leishmania sp. from the tuberculoid leprosy type of cutaneous leishmaniasis from Ethiopia gives the greatest number of arcs with antisera to a strain of L. tropica. The antigen present in the fluid phase of NNN medium is also present in the flagellates as common arcs are formed between it and both anti-flagellate serum and immune monkey serum. It cannot therefore be properly termed an exo-antigen.

The Leishman-Donovan (LD) bodies of L.11 L. mexicana both from hamsters and from tissue cultures were also used in the test. They gave more arcs to sera from disseminated or visceral L. mexicana infection than did the leptomonad antigen, but fewer arcs to sera from kala-azar patients.

DISCUSSION

The evidence, based upon the number of precipitation arcs, their intensity and their identity, points to several indications which are however, no more than indications:

a) it seems that L. mexicana and the Leishmania sp. from Costa Rica and Panama are distinct, as in double diffusion tests they share only two antigens out of four demonstrated, and L. mexicana antigen fails to react with immune sera from costa rican patients. This indication is conclusively borne out by the work of LAINSON and SHAW (1966) who showed that L.
*mexicana* infection in man failed to immunize against panamanian cutaneous leishmaniasis;

b) *L. mexicana* and *L. tropica* share only one antigen among four or five demonstrated in the test despite the cross-immunity between the two diseases (ADLER and GUNDER, 1964)

c) there remains little evidence still that our L15 strain from a “diffusa” patient in Belém, Brazil, is anything more than an unusually virulent form of *L. braziliensis*;

d) *L. donovani* strains usually share three or more common antigens with all the strains at our disposal;

e) the strain of *Leishmania* sp. from Ethiopia, causing skin lesions resembling tuberculoid leprosy, shares the most common antigens with *L. tropica*;

f) there is some evidence that LD body antigen may be more specific than leptomonad antigen in tests with immune sera from patients or infected animals;

g) lastly there is evidence that each of the strains used here is antigenically distinct, regardless of species, and it would appear to be impossible to draw definite conclusions about the status of species of *Leishmania* from evidence supplied by double-diffusion tests.

There remains the fact that in many cases antigen-antibody precipitation arcs shared by two antisera and a single antigen may not be shared by a third antiserum and the same antigen. It is also obvious that there usually exists one arc which is peculiar to a homologous system but is absent from heterologous systems. In order to characterize these reactions, further work on electrophoresis of the antigens is in progress. This is especially necessary as in the profusion of arcs obtained there is obvious evidence of interference and deviation, which is impossible to interpret where so many arcs are present.
REFERENCES


