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Identification and taxonomy of human and animal leishmanias by lectin-mediated agglutination

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Summary

The surface polysaccharides of two strains of lizard leishmanias, of the guinea pig parasite *L. enrietti* and of nine strains of human leishmanias belonging to the groups *mexicana*, *donovani* and *tropica* were studied by lectin-mediated agglutination. Twenty-three lectins prepared from seeds or from higher fungi carpophores were used. They revealed the presence of L-fucose, N-N′-diacetylchitobiose, α-D-glucose, α-D-mannose, β-D-galactose, N-acetyl-D-galactosamine and lactose. We noted a range in the number and variety of lectin receptor sites detectable on the surface of the leishmanias, with the reptiles strains having the fewest sites and the *tropica* group having the most sites. We were unable to find specific group lectins, but it seems possible to identify a strain within each group by a lectin-binding pattern.

Key words: *Leishmania*; identification; taxonomy; lectin.

Introduction

Research is still being done on the genus *Leishmania*, partly because of the great morphological similarities found in the promastigote and amastigote stages. Also, the clinical characteristics and the geographical distribution used for genus classification have proven insufficient because identical clinical pictures are encountered in widely distant geographical areas. For this reason a large number of studies have been carried out to find other criteria for the identification of strains and for the definition of species and sub-species.

The main methods used have been the study of ultrastructural morphological (Lewis, 1975; Shaw and Lainson, 1976; Gardener et al., 1977) or biochemi-

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cal characteristics with kinetoplastic and nuclear DNA buoyant densities (Chance et al., 1974; Barker and Arnot, 1981; Arnot and Barker, 1981), the electrophoretic variation of different enzymes (Ebert, 1973; Gardener et al., 1974; Kilgour et al., 1974; Al-Taqui and Evans, 1978; Chance et al., 1978; Rassam et al., 1979; Kreutzer and Christensen, 1980), the comparison of histones (Icekson and Schnur, 1980), and radiorespirometry (Decker et al., 1977). Immunological techniques utilizing determination of serotypes specific to excreted elements (EF, Excreted Factor) (Schnur et al., 1972; Schnur and Zuckerman, 1977), immunoelectrophoretic analysis (Matossian-Rogers et al., 1976; Kohanteb et al., 1980; Le Ray and Afchain, 1980), and use of monoclonal antibodies (MacMahon Pratt and David, 1981) have produced further information. More recently, lectins have been used to explore membrane structures; these vegetable proteins link with surface sugar residues (Lis and Sharon, 1977; Goldstein and Hayes, 1978; Goldstein et al., 1980) and can be used to separate and identify strains of protozoa (Bretting and Schottelius, 1978; Pétavy et al., 1978; Gueugnot et al., 1980; Gueugnot, 1980; Araujo et al., 1980) including the leishmanias (Jacobson et al., 1982; Schottelius and Gonçalves da Costa, 1982; Schottelius, 1982).

In the present work, 13 strains of human or animal origin were studied using 23 lectins original in the majority of cases.

Materials and methods

Strains

The strains used in this study were as follows:

Peripylaria Section (Lainson and Shaw, 1979):

- *Leishmania adleri* LRC-L123: a strain isolated in Kenya from a lizard *Lastatia longicauda revoili* by Heisch in 1954. Injected into hamsters by Adler, this strain was again isolated in 1956 from the spleen of one of these rodents. It was given to the Institute of Tropical Medicine of Anvers (ITMA) by the London School of Tropical Medicine and Hygiene.
- *Leishmania tarentolae senegalensis* G.10: a strain isolated by Ranque in Senegal from a *Tarentolae annularis* in 1967.

Suprapylaria Section (Lainson and Shaw, 1979):

- *Leishmania mexicana mexicana* London L11: a strain isolated in the Honduras from a human case.
- *Leishmania mexicana amazonensis* LRC-L259 (= LVI60 = M2269): a strain isolated in Brazil from a human cutaneous lesion.
- *Leishmania* sp. (L.sp.): a strain isolated in 1960 from a human cutaneous lesion in Salvador de Bahia (Brazil). It was given to the ITMA in 1963.
- *Leishmania enrietti:* a strain isolated in Brazil from a guinea pig (genus *Cavia*) then maintained at the Institut Pasteur in Paris. It was received by the ITMA in 1963.
- *Leishmania donovani* ITMAP K263: a strain isolated in Brussels in 1967 by the seeding on blood agar of the sample from a sternal puncture carried out on a Moroccan child.
- *Leishmania donovani* Tunis: a strain isolated in Tunisia from a human case, which was received by the ITMA in 1965.
- *Leishmania donovani* LRC-L133: a strain isolated in Ethiopia from a human Kala-azar case.

- *Leishmania aethiopica* LV1: a strain isolated from a human cutaneous lesion, sent by Bray to Liverpool and numbered L86 in the London collection of *Leishmania.*
- *Leishmania tropica* LV556 (= LRC-L36): a strain isolated from a cutaneous lesion acquired in Iraq.
- *Leishmania tropica* LRC-L32: a strain isolated at Jerusalem in 1965 from an Iraqi immigrant, who was a typical case of leishmaniasis recidivans.
- *Leishmania tropica* LRC-L223: a strain isolated in Israel from a simple cutaneous leishmaniasis.

The parasites were cultivated in a Brain-Heart Infusion Medium (Difco) with the addition of 3% defibrinated rabbit blood at a temperature of 27–28°. The promastigotes were collected by centrifugation (1200 $\times g$, 5 min) after a period of cultivation of 96 hours and washed twice in a phosphate buffer pH 7.2 (PBS). They were then resuspended in the same buffer at 1.5×10^5 promastigotes $\cdot ml^{-1}$.

Lectins

Twenty-three lectins were used for this study. They were purified in the laboratory, using different affinity chromatography techniques, from seeds or from higher fungi carpophores, except for fractions of *Ricinus communis* RCA_{120} and RCA_{60} (IBF). These lectins are grouped together in Table 1. For each lectin the following data are given:

1. the haemagglutinating activity obtained by serial 2-fold dilution with a suspension of red blood cells from blood group A (4% in PBS);
2. the main inhibiting polysaccharide(s) obtained by tests on the inhibition of agglutination of red blood cells of blood group A and of various *Trypanosomatidae* (Gueugnot, 1980);
3. purification reference.

All the lectins were used in a phosphate buffer (PBS) at 500 $\mu g \cdot ml^{-1}$; for fluorescence tests the lectins were linked with fluorescein using Goldman's method (Goldman, 1968).

Agglutination

The agglutination tests were carried out in microtiter plates by mixing 0.1 ml of promastigote suspension ($1.5 \times 10^5 \cdot ml^{-1}$) with 0.1 ml of lectin solution (500 $\mu g \cdot ml^{-1}$). After one hour of contact at room temperature, agglutination was read under the microscope ($\times 100$), estimated at 0 to 4+ and compared with a control sample where lectin was replaced by PBS. The shape and the size of the agglutinates as well as any possible toxic effects were observed at the same time ($\times 400$).

Inhibition of agglutination

The sugars used for the inhibition of agglutination are those shown in Table 1 and have the following concentrations: 0.1 M for the lactose and the N-N'-diacetylchitobiose, 0.2 M for the other sugars. These experiments were also carried out in microtiter plates. $1/10$ ml of sugar solution and 0.1 ml of lectin solution were incubated for 30 minutes at room temperature before addition of the promastigotes. The agglutination reading was made after an hour of contact under the conditions given above.

Fluorescence tests

The binding sites were visualised by the use of fluorescent lectins. A drop of fluorescent lectin solution was added to one drop of protozoa suspension placed between two slides on the side of the cover slide. One examination was made immediately and another after half an hour of contact.

Results

The results of the agglutination tests are summarized in Table 1. All the experiments have been repeated three times at least. The lectin-mediated ag-

Table 1. List of lectins used and results of experiments of agglutination of animal and human leishmanias

Lectins	TH	Inhibiting sugars	Ref.	L.adl.	L.tar.
Ulex europaeus I	1/64	L-fucose	Bétail et al., 1975	0	0
Laccaria amethystina F	1/64	L-fucose	Gueugnot, 1980	0	0
Datura stramonium	1/512	N-N′-diacetylchitobiose	Bétail et al., 1975	0	0
Ulex europaeus II	1/256	N-N′-diacetylchitobiose	Gueugnot, 1980	0	0
Lathyrus odoratus	1/128	α-D-glucose	Bétail et al., 1969	+	+
Pisum sativum	1/128	α-D-glucose	Bétail et al., 1969	+	+
Lens esculenta	1/128	α-D-glucose	Bétail et al., 1969	+	+
Canavalia ensiformis	1/512	α-D-methyl-mannoside	Bétail et al., 1969	+ + + +	+ + + +
Boletus edulis	1/1028	β-D-galactose	Gueugnot, 1980	+	0
Sarothamnus scoparius	1/1028	β-D-galactose lactose	Bétail et al., 1975	+ + +	+ + + +
Boletus subtomentosus	1/64	β-D-galactose lactose	Gueugnot, 1980	0	+
Pholiota squarrosa	1/1028	β-D-galactose lactose + N-acetyl-D-galactosamine	Bétail et al., 1975	+	+ + +
Soja hispida	1/8	β-D-galactose lactose + N-acetyl-D-galactosamine	Bétail et al., 1975	+ +	+
Clitocybe nebularis	1/512	β-D-galactose lactose + N-acetyl-D-galactosamine	Gueugnot, 1980	+ + + +	+ + + +
Ricinus communis 60	1/64	N-acetyl-D-galactosamine	IBF	+ + + +	+ + + +
Ricinus communis 120	1/4048	Lactose	IBF	+ + + +	+ + + +
Sophora japonica	1/256	Lactose	Bétail et al., 1975	+ + +	+ +
Polyporus sulfureus	>1/4000	Lactose	Gueugnot, 1980	+ +	+
Inocybe fastigiata	1/1028	Lactose	Gueugnot, 1980	+ +	+
Laccaria amethystina L	>1/4000	Lactose	Gueugnot, 1980	0	0
Boletus chrysenteron	1/128	Lactose	Gueugnot, 1980	+ + +	+ + +
Cytisus albus	1/128	Lactose D-salicine	Bétail et al., 1975	0	+
Phaseolus vulgaris	>1/4000	N-acetyl-D-galactosamine	Bétail et al., 1975	+	0

Lizard leishmanias: *L. adleri:* L.adl. – *L. tarentolae:* L.tar. *Guinea pig leishmania: *L. enrietti:* L.enr.
mexicana group: *L. m. amazonensis* LRC-L259: L.259 – *L. m. mexicana* London L11: L.11 – L.sp. from a cutaneous lesion (Brazil)
donovani group: *L. donovani* Tunis: L.Tu. – *L. donovani* LRC-L133: L.133 – *L. donovani* ITMAP K263: L.K263
tropica group: *L. aethiopica* LVI: LV1 – *L. tropica* LCR-L223: L.223 – *L. tropica* LV556: LV.556 – *L. tropica* LRC-L.32: L.32

glutination was estimated and compared with a control sample, when a weak auto-agglutination occurred, it was taken into account.

With the wide range of lectins, L-fucose, β-D-galactose, N-acetyl-D-galactosamine, lactose, α-D-glucose, α-methyl-D-mannoside, and N-N′-diacetylchitobiose sites were determined. Lectins of *Ricinus communis* RCA_{120}, *Canavalia ensiformis* and *Boletus chrysenteron* agglutinate all strains. *Datura stramonium*, on the other hand, a lectin inhibited in a specific manner by N-acetyl-D-glucosamine and its oligomers (Crowley and Goldstein, 1981) has practically no agglutinating effect on any of the strains. *Ulex europaeus* II however, which has the same specificity (Goldstein and Hayes, 1978), binds to several strains. Different types of agglutination were observed: cell body-cell body, cell body-flagellum, flagellum-flagellum.

by 23 lectins purified from seeds or from high fungi carpophores

L.enr.	L.259	L.11	L.sp.	L.Tu.	L.133	L.K263	LV1	L.223	LV.556	L.32
0	0	0	+	0	++	0	0	+	+++	++++
+++	0	0	0	0	0	+++	0	0	+++	++
0	+	0	0	+	0	0	0	0	0	0
0	0	+	0	+	0	++++	0	++++	++++	+++++
+	0	+	0	+	0	+	++	++	+++	0
+	+++	+	+	+++	++++	++++	+++	++	+++	+++++
0	+++	+++	+	+++	++	++++	+++	++	+++	++
+++++	+++++	+++	++++	+++++	+++++	++++	+++	+++	+++	++++
+++	+++++	0	++++	+++++	+++++	++++	++	+++	++++	++++
++++	0	+++	++++	+++++	+++++	++++	++	++++	++++	++++
0	+	0	++	0	++++	++	+++	+++	++++	++++
0	0	0	0	+	+++	+	+++	++++	++++	+++
++	0	0	++	+++	+++	++++	++	+++++	+++++	+++++
++++	0	0	++++	+++++	+++	++++	+++++	+++++	+++++	+++++
++++	0	++++	+++++	+++++	++++	+++	+++	+++++	+++++	+++++
++++	+++++	++++	+++++	+++++	++++	++++	+++++	+++	++++	++++
++++	0	+	+++	+++++	0	++++	++	+++++	+++	++++
++++	0	++	+++	+++++	++++	++++	0	+++++	+++	++++
++++	+++++	0	++++	+++++	++++	++++	++	+++++	+++	++++
+++	+++++	0	0	0	+	++++	0	++	++++	++++
+++	+++++	+++	+++++	+++++	++++	++	+++++	++	+++++	+++++
++	0	++	0	0	0	++++	0	++++	++++	+++++
+	0	0	+	0	++	0	0	0	++	+

In most cases it was possible to inhibit agglutination by the addition of specific sugar(s) in the reaction mixture. The use of non-specific sugars produced no notable effect.

The results of fluorescence tests agreed with those of agglutination. We were able to observe differences in the localisation of lectin-binding sites. Some bind in a uniform manner with the whole parasite (cellular body, flagellar pocket, flagellum) while others recognise sites only on the cell body.

Discussion

Mammalian leishmanias

On the whole, isolated mammal strains offer a great variety of receptor sites for the lectins used.

We can relate *L. enrietti*, isolated from a laboratory guinea pig, by its behaviour towards lectins, to leishmanias of the *mexicana* group and in particular to *Leishmania* sp. A striking difference however, is the presence in *L. enrietti* of binding sites for the *Laccaria amethystina* lectin inhibited by L-fucose. Lainson and Shaw (1972) had also noted that despite the greater size of the amasti-

gote stage, *L. enrietti* could be related to leishmanias of the *mexicana* group by its growth characteristics in NNN medium and by its pathogenic behaviour towards the guinea pig. What is more, the same authors include *L. enrietti* in the *mexicana* complex of the Suprapylaria section of their most recent classification (Lainson and Shaw, 1979).

The *Pholiota squarrosa* lectin which is consistently active on the Suprapylaria leishmanias of the Old World, although to different degrees, is inactive on strains of the *mexicana* complex. *Mexicana* strains are agglutinated by the other lectins in a non-identical fashion. *L. m. mexicana* and *L. m. amazonensis* can be differentiated by seven lectins which have in common a sensitivity to β-D-galactose (*Boletus edulis*, *Sarothamnus scoparius*, RCA_{60}, *Polyporus sulfureus*, *Inocybe fastigiata*, *Laccaria amethystina* lactose, and *Cytisus albus*).

Moreover *L. m. amazonensis* appears to have appreciably more sites of type α-D-glucose but fewer sites of type β-D-galactose. It is also interesting to note that, on the one hand, *L. m. amazonensis* is the only strain of leishmania studied which is not agglutinated by the lectins RCA_{60} and *Sarothamnus scoparius*, and on the other hand that *L. m. mexicana* is not agglutinated by the lectin of *Boletus edulis*, unlike the other mammalian strains. The non characterized strain, *Leishmania* sp., isolated in Brazil, possesses an appreciably higher number of receptor sites and also has agglutination reactions specific to it. We found the same activity as Jacobson et al. (1982) and Schottelius (1982) for Ricin RCA_{120} and for concanavalin A on *L. m. mexicana*. Using a range of 12 lectins, Schottelius (1982) was able to determine two groups within the subspecies *L. m. amazonensis* and noted the similar behaviour of a strain of *L. m. pifanoi* with one of these two groups.

The leishmanias which react with the greatest number of lectins belong to the complex of the Old World of the section Suprapylaria, the *tropica* complex being the most easily agglutinated.

For the *donovani* species, as well as for the *mexicana* complex, we noted a behavioural inconsistency within the species since the three strains can be separated by use of 9 lectins (*Laccaria amethystina* fucose, *Ulex europaeus* I and II, *Boletus subtomentosus*, *Pholiota squarrosa*, *Sophora japonica*, *Laccaria amethystina* lactose, *Cytisus albus*, *Phaseolus vulgaris*). Strain LRC-L133, which belongs to the serotype of Excreted Factor B_2, reacts with Soja and *Phaseolus vulgaris:* Jacobson et al. (1982) however noted the absence of reactivity to these lectins by this and other strains of the same serotype. This discrepancy may be due to the difference in concentration of the lectins used.

In the *tropica* complex, *L. aethiopica* shows a behaviour which allows it to be easily separated from other members of the complex that we have studied. *Polyporus sulfureus, Cytisus albus* and *Laccaria amethystina* specific for lactose, as well as *Ulex europaeus* II specific for N-N′-diacetylchitobiose do not agglutinate this strain. Schottelius (1982) also noticed the lesser density of sugar residues on its surface membrane. In support of these results, results based on ultra-

structural study and on kinetoplastic and nuclear DNA buoyant densities show that *L. aethiopica* had a separate place in the complex (Barker and Arnot, 1981; Arnot and Barker, 1981). The other strains are closely related. However strain LRC-L223 differs from LV556 and LRC-L32 by the absence of L-fucose residues. LRC-L32 possesses no sites for *Lathyrus odoratus* lectin. For this strain of serotype of Excreted Factor A_2, our results are in agreement with those produced by Jacobson et al. (1982).

Lizard leishmanias

The lizard leishmanias *L. tarentolae* and *L. adleri* show a clear difference from the other strains studied, whether they be of human origin or isolated in the guinea pig, by the relatively weak density of the detectable receptor sites. We found no L-fucose residue on their surface; there is little reactivity for the D-glucose specific lectins nor for D-mannose sites, with the exception of concanavalin A. However, a higher density of receptor sites for lectins with a sensitivity to D-galactose was found. Four lectins in particular have a strong agglutinating action on them (*Sarothamnus scoparius, Clitocybe nebularis,* RCA^{60} and RCA^{120}). Thus the reaction pattern of lizard leishmanias is not superimposable on any of the others. This seems to be confirmed by morphological (Gardener et al., 1977) and immunological studies (Saf'janova and Avakjan, 1973), which have already shown that there are great differences between the lizard leishmanias and leishmanias isolated from man. Adler (1964), Gardener et al. (1977) and Saf'janova and Avakjan (1973) have put forward the hypothesis that *L. adleri* might represent an evolutionary link between reptile and mammal species. The similar reactivity of *L. adleri* and *L. tarentolae* does not allow us here to confirm this hypothesis since only one lectin *Pholiota squarrosa* enables us to differentiate them clearly.

Within the limit of the strains studied, examination of the results of agglutination by the lectins used here shows an increasing density of surface receptor sites from the lizard leishmanias to leishmanias of the *tropica* complex. Moreover, this result emphasises the complexity of the genus *Leishmania*, since we were unable to determine the existence of lectins specific to each group; on the contrary, there was a behavioural inconsistency within each group. These results are supported by the observations of Lainson and Shaw who feel that there is no clear division between the leishmanias of the Old and New World on the one hand, or between the leishmanias responsible for cutaneous affections and those responsible for visceral affections (Lainson and Shaw, 1979). As Gardener et al. (1974) stress in connection with the treatment of the data concerning the biochemical and immunological characteristics, we seem to be leading towards an identity card of the strain rather than towards a taxonomy as it is understood by botanists and zoologists.

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