TARGET ORIENTED DRUGS AGAINST LEISHMANIA. (U)

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REPORT NUMBER 2

TARGET ORIENTED DRUGS AGAINST LEISHMANIA

(Second Annual Summary Report)

URI ZEHAVI, Ph.D.

and

JOSEPH LL-UN, Ph.D.

supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-30-G-9474

(previously, Contract No. DAMD 17-75-G-5452)

Faculty of Agriculture
The Hebrew University of Jerusalem
P.O. box 12, Rehovot 76-100, Israel

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.
**Abstract**

1. Excreted Factor (EF) is a carbohydrate-rich protein excreted by different strains of *Leishmania*. It has antigenic properties similar to those of the parasite and plays a role in the infective process.

2. Isolation and purification of EF is necessary for:
   - (a) study of its biological function
(b) the use of LF for diagnostic purposes
(c) the use of LF in immunization experiments
(d) the study of the biosynthetic steps of LF
(e) the preparation of inhibitors of particular biosynthetic steps of LF.

3. Purification of LF by affinity chromatography was markedly improved by introducing 
Racemus lectin (specific for galactose) column. This enabled us to obtain more reliable amino acid and sugar analysis and will be instrumental in more advanced physical, chemical and immunological studies.

4. We have developed a radioimmunoassay for leishmaniasis utilizing purified LF. The assay can distinguish between Leishmania strains and once further developed, should prove most valuable for the diagnosis of the disease.

5. LF plays a role in the infective process of Leishmania. We have now shown that surface carbohydrate, related to LF, plays a role in the initial attachment of Leishmania promastigotes to macrophages - a stage that is a prelude to their engulfment by the macrophages followed by multiplication in their cells.
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2. Isolation and purification of EF is necessary for:
   (a) study of its biological function
   (b) the use of EF for diagnostic purposes
   (c) the use of EF in immunization experiments
   (d) the study of the biosynthesis of EF
   (e) the preparation of inhibitors of particular biosynthetic steps of EF.

3. Purification of EF by affinity chromatography was markedly improved by introducing *Acanthopanax lectin* (specific for galactose) column. This enabled us to obtain more reliable amino acid and sugar analysis and will be instrumental in more advanced physical, chemical and immunological studies.

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2. Purification of EF</td>
<td>4</td>
</tr>
<tr>
<td>3. Chemical Analysis of EF Preparations</td>
<td>4</td>
</tr>
<tr>
<td>a) Amino Acid Analysis - Table 1</td>
<td>5</td>
</tr>
<tr>
<td>b) Monosaccharide Analysis - Table 2</td>
<td>6</td>
</tr>
<tr>
<td>4. Radioimmunoassay (RIA) for the Diagnosis of Leishmania</td>
<td>6</td>
</tr>
<tr>
<td>5. Surface Carbohydrates (Related to EF) and the binding of Leishmania Promastigotes to Macrophages</td>
<td>10</td>
</tr>
<tr>
<td>6. Conclusions</td>
<td>11</td>
</tr>
<tr>
<td>7. Proposals for Further Research</td>
<td>11</td>
</tr>
<tr>
<td>8. Figures</td>
<td></td>
</tr>
<tr>
<td>a) Figure 1</td>
<td>12</td>
</tr>
<tr>
<td>b) Figure 2</td>
<td>13</td>
</tr>
<tr>
<td>c) Figure 3</td>
<td>14</td>
</tr>
<tr>
<td>d) Figure 4</td>
<td>15</td>
</tr>
<tr>
<td>9. Literature Cited</td>
<td>16</td>
</tr>
<tr>
<td>10. Glossary</td>
<td>17</td>
</tr>
<tr>
<td>11. Distribution list</td>
<td>17</td>
</tr>
</tbody>
</table>
1. Introduction

Excreted Factor (LF) is a carbohydrate-rich protein excreted by different strains of *Leishmania*. Our first annual report has demonstrated the presence of very high galactose content in LF, a property that could be shared also by the surface carbohydrates of the parasite. LF has immunological properties similar to those of the intact parasite and may play a role in the infective process. After it was shown to be species-specific, LF became the basis for serotyping *Leishmania* strains, and is a valuable tool in diagnostic, demographic and ecological studies of the disease (2). The diagnostic methodology, however, is generally insufficient and the development of a reliable radioimmunoassay (RIA) seems most valuable.

2. Purification of LF

LF is produced in relatively small quantities by *Leishmania* promastigotes in culture. Purified LF was required for (a) RIA development, (b) attachment studies and (c) chemical and physical analysis. More advanced chemical and physical studies were therefore hampered by difficulties to scale up the isolation of LF from culture media. This obstacle is now more successfully overcome by directing additional technical help to this issue.

(i) Isolation and phenol extraction - *L. tropica* and *L. donovani* promastigotes were cultivated in Lit medium supplemented with 10% foetal calf serum and antibiotic 100 μg streptomycin and 100 μg penicillin per ml. Cultures at the logarithmic phase of the growth were centrifuged 7 min at 1500 ×g. The supernatant was removed and concentrated to 1/10 the original volume and dialyzed for 3 days against distilled water. The dialyze was concentrated again and extracted with phenol. The aqueous phase was dialyzed for 3 days against distilled water and the dialyzed concentrated LF was fractionated on Sephadex G-100 column. The fractions containing LF as detected by immunodiffusion were pooled, concentrated by freeze-drying and dialyzed again for 3 days against saline. This procedure represents a refinement of that described in the first annual report, 14a.

(ii) Affinity chromatography (Ricinus lectin column)

In a typical experiment, 16 mg of LF (14b) were applied to a Ricinus lectin column (Agarose-Ricinus communis Agglutinin 120, 1.1 mg protein per ml resin, 7 cm. long, 1 cm. in diameter). The column was first eluted with 0.02 M sodium phosphate buffer, pH 7.0 (12 fractions) followed by a similar buffer containing 50 μM galactose (all fractions contained 2.6 ml per fraction). The fractions containing LF were detected by immunodiffusion (fraction 14-16), pooled, dialyzed against water and lyophilized to yield purified LF (2.0 mg) possessing 6 x increase in the specific antigenic activity.

This technique appears to be fast and more efficient than previous techniques for the purification of LF.

3. Chemical Analysis of LF Preparations

Amino acid analysis and monosaccharide analysis were carried out on
samples of EF, purified by affinity chromatography (521) following acid hydrolysis or methanolysis and trimethylsilylation, respectively.

(a) Amino acid analysis - EF L137 was analyzed following acid hydrolysis using amino acid analyzer (LKU Model 3201). See Table 1 for results.

Table 1
Amino acid analysis of EF L137

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar %a</th>
<th>Relative number of amino acid residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>5.8</td>
<td>1.65</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.16</td>
<td>0.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.83</td>
<td>0.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.29</td>
<td>2.07</td>
</tr>
<tr>
<td>Threonine</td>
<td>22.99</td>
<td>6.55</td>
</tr>
<tr>
<td>Serine</td>
<td>16.59</td>
<td>4.73</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.57</td>
<td>2.73</td>
</tr>
<tr>
<td>Alanine</td>
<td>10.35</td>
<td>2.95</td>
</tr>
<tr>
<td>Half cystine</td>
<td>13.54</td>
<td>3.66</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.51</td>
<td>1.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.13</td>
<td>1.18</td>
</tr>
<tr>
<td>Glucosamine</td>
<td></td>
<td>4.76</td>
</tr>
<tr>
<td>Galactosamine</td>
<td></td>
<td>1.01</td>
</tr>
</tbody>
</table>

a Total not including glutamic acid, proline and hydroxyproline amounting to 13.44% (not separable in this run).

In the first annual report, 55a, we have commented on the particular features of the amino acid analysis. Here we find some deviations obviously related to the (ca.b x) purification that we have achieved. Characteristically, aromatic amino acids are entirely absent.

(b) Carbohydrate analysis - Monosaccharide components of EF were determined by GLC following methanolysis and trimethylsilylation (3).
Table 2

Monosaccharide composition of various EFs

<table>
<thead>
<tr>
<th>Sugar</th>
<th>EF L137</th>
<th>EF L52</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rel. to mannose</td>
<td>rel. to mannose</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Xylose</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Galactose</td>
<td>4.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Total sugar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sugar by Dubois</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Amount relative to mannose (Man = 1.0).

^b Glucosamine and galactosamine are presented in Table 1.

^c A large proportion of the xylose and some of the glucose may represent a contaminant eluted from the affinity column.

Fucose, a monosaccharide frequently present at the non-reducing end of glycoproteins and glycopeptides was determined here for the first time.

The proportion of arabinose has diminished as the result of the present purification.

Galactose remains the most abundant neutral sugar.

Microheterogeneity, demonstrated, for instance, by the increase of galactose/mannose ratio if EF peaks between early and late fractions can be understood in view of the galactose binding properties of the Ricinus lectin column (the sequence of fractions I, II, III).

4. Radioimmunoassay (RIA) for the diagnosis of Leishmaniasis

(a) Labeling by ^3^H-Acetic Anhydride

EF L137 partially purified by phenol extractions and followed by Sephadex
column chromatography (§2i) was acetylated under Schotten-Baumann conditions (acetic anhydride with acetic anhydride) to yield high specific activity labeled EF. Although most of the label precipitated with homologous rabbit anti-
serum, further column chromatography (§2ii) separated the label from the immunological activity. Thus, the entity labeled must have been a peptide component still attached to, but separable from, EF.

(a) labeling by galactose ester - sodium borotritiate

EF (§2i) was labeled in the non-reducing terminal galactosyl residues by the galactose ester-sodium borotritiate technique (4). A crude sample of labeled EF (1,500 CPM) was added to a carrier EF (§2i), 12 mg and was further purified by affinity chromatography (§2ii). The purified EF contained 5,500 CPM.

RIA employing 125I-protein A

This technique, originally developed in parallel to the labeling procedure described in §6a and §6b has the advantage of using harder radiation (125I vs. 3H) and developed faster into a useful RIA.

The technique, based on the coating of antibody to solid phase coating (5), followed by protein A labeling, was used as previously by Avraham et al. The test was done in both polystyrene tubes (100mm x 13mm, screw cap) and 96 wells microplates (Sterilin, England). Coating of the wells with tubes or microplates either treated with glutaraldehyde or left untreated, 0.1M and glutaraldehyde were diluted in water buffer saline (BBS) 1/8 and dilutions of sera and protein A were made in BBS containing the fetal calf serum. The assay for avidin binding and its inhibition of EF was as follows: 100 µl of EF at different concentrations were added to the 96 wells of microplates, which had been treated or not treated with 0.1% glutaraldehyde. After 24 hr incubation at 4°C, different dilutions of antisera were added. After another 24 hr incubation at 4°C, the unbound antibodies were removed and 100 µl 125I-protein A (specific activity about 50,000 counts/min) were added. The reaction was terminated by the addition of 20 µl of 0.15M NaCl. The solution from each well was then transferred to plastic tubes and the radioactivity measured.

For inhibition studies, 0.2 µl of 1:10 dilutions of antisera were mixed with serial dilutions of EF at a final volume of 0.2ml. After incubation for 30 min at 37°C and centrifugation at 4°C, the adsorbed sera were added to the EF coated microplates and the radioactivity was measured.

Label carriers and other criteria are as follows:

(b) coating tubes

For coating tubes, pre-coat 96-well microplates by gel filtration on Sephadex G-100 (§2i) and to be suitable for binding to tubes and microplates wells.

The preparations obtained by either precipitation and chromatography or by the dissociation of immune complexes, did not bind to the tubes and showed the same activity with antibody as untreated.
control EF. It appears that the absolute amount of EF coupled
to the plate depends essentially on the level of purification and the
nature of the medium component which acts as a carrier rather
than the amount of antigenic determinants of the EF.

The concentration of EF used for coating the wells was 0.6 to 1.2
mg/ml. Immunodiffusion of this EF with homologous antiserum pro-
duced an immune precipitate at a maximum dilution 1:2. Concentra-
tions exceeding 1.2 mg/ml increased the strength of the binding
without affecting the specificity (Fig.1). At concentrations less
than 0.35 mg/ml, no antibodies were detected. Almost no differences
in extinction values were seen with wells coated or uncoated with
Dialyzed.

Specificity of the assay

The interaction of antisera with different EFs and the specificity
of the reaction is given in Fig. 1. The results obtained indicated
that anti-L. tropica and anti-L. donovani reacted only with homolo-

gous EF's, owing to the higher concentration of rabbit anti-L. dono-

vani, Inc., as measured by immuno-diffusion, higher binding was observed
with this antiserum, as compared with anti-L. tropica serum at

corresponding dilutions.

In most cases, the activity obtained with the heterologous antiserum
was almost the same as that of control normal rabbit serum. With
antisera diluted at 1:10, 10% of the anti-L. donovani were bound to
EF with tropica EF, 32% of anti-L. tropica antibodies were bound to L.

Donovani EF and 12.5% to 16% of normal rabbit antibodies were bound

cross-specifically to L. donovani and L. tropica EF respectively.

Neutralization study

As indicated in Fig. 1, the reaction of both anti-L. donovani and
anti-L. tropica sera could be completely inhibited by preincubation
with homologous EF. 300 µg of L. donovani and L. tropica EF were
sufficient to block all the anti-EF antibodies present in 1 ml of
heterologous antiserum.

Titration of anti-EF antibodies in mice and humans suffering from

cutaneous leishmaniasis

Anti-EF activity in sera from humans infected with L. tropica major
is shown in Table 3. Of 7 sera examined from patients with active
cutaneous leishmaniasis, 5 (71%) showed maximum activity of 1.4 to

2.5 times higher than normal uninfected control.

Serum collected from both C mice 40 days after infection with L.
tropica, was also assayed for anti-EF antibodies. These mice
were autopsied, well-developed lesions and parasites were detected in both,
their lesion and in their spleens. The anti-EF antibody activity
detected in these sera was as high as 25.6 times that of a normal
non-infected mouse serum.
Table 3. Anti-\textit{L. tropica} 2F antibody activity in sera from patients with cutaneous leishmaniasis.

The numbers in brackets are the results compared with the activity measured in normal human serum.

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Serum A</th>
<th>Serum B</th>
<th>Serum C</th>
<th>Serum D</th>
<th>Serum E</th>
<th>Serum F</th>
<th>Serum G</th>
<th>Control serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>42.3(12.19)</td>
<td>17.2(9.89)</td>
<td>13.3(0.68)</td>
<td>13.6(0.7)</td>
<td>22.9(1.18)</td>
<td>21.4(1.10)</td>
<td>22.3(1.15)</td>
<td>19.3</td>
</tr>
<tr>
<td>1:10</td>
<td>22.8(2.25)</td>
<td>15.6(1.48)</td>
<td>9.3(0.92)</td>
<td>9.9(0.98)</td>
<td>19(1.9)</td>
<td>14.2(1.40)</td>
<td>10.5(1.63)</td>
<td>10.1</td>
</tr>
<tr>
<td>1:25</td>
<td>13.0(2.13)</td>
<td>9.0(1.47)</td>
<td>6.2(1.0)</td>
<td>5.5(0.9)</td>
<td>11.2(1.83)</td>
<td>6.0(1.08)</td>
<td>10.7(1.75)</td>
<td>6.1</td>
</tr>
<tr>
<td>1:50</td>
<td>8.3(1.55)</td>
<td>7.9(1.19)</td>
<td>4.4(0.66)</td>
<td>3.9(0.39)</td>
<td>6.7(1.01)</td>
<td>4.0(0.4)</td>
<td>5.3(0.8)</td>
<td>6.0</td>
</tr>
<tr>
<td>1:100</td>
<td>5.7(1.03)</td>
<td>4.7(0.85)</td>
<td>3.4(0.61)</td>
<td>2.8(0.56)</td>
<td>4.0(0.83)</td>
<td>2.0(0.47)</td>
<td>3.3(0.6)</td>
<td>5.5</td>
</tr>
</tbody>
</table>
The immunological specificity of leishmanial excretory factors, and their separation from other leishmanial antigens and contaminating medium components were used to provide a more sensitive means of detecting and measuring the antibody response to leishmanial infection. The results obtained indicated that the use of leishmanial LF for RIA will prove valuable in the diagnosis of leishmaniasis. The test is specific and sensitive. Undoubtedly, experience will lead to modification and refinement of the technique according to its specific application. However, sufficient details have been presented to enable its use with regard to the detection of cutaneous leishmaniasis and quantitative measurement of LF levels.

5. Surface Carbohydrate (Related to EF) and the Binding of Leishmania Promastigotes to Macrophages

It is known that liver and peritoneal macrophages have specific galactose-binding receptors (hepatic binding protein, HBP) which are able to bind neuraminidase-treated cells where galactose is the non-reducing sugar as a prelude to their catalysis (6). It was demonstrated recently, however, that the galactose or the N-acetyl-galactosamine specificity of HBP is not high and additional monosaccharides (e.g. glucose) can compete, though less favourably, for HBP binding (6a).

The first annual report, §5b, has described the presence of a high galactose content in EF, a finding that was further supported by our current analyses, §5b. In addition, we have demonstrated that promastigotes of both L. tropica and L. donovani are agglutinated by low concentrations of peanut lectin and Ricinus lectin and that dissociation is readily achieved by the addition of galactose. This indicates the presence of oligosaccharides possessing terminal non-reducing galactose as a partial structure of the promastigote surface (first annual report §2a and current results concerning Ricinus lectin).

We propose that the host cell (macrophage) membrane might contain a galactose-binding receptor, possibly the well-documented hepatic binding protein (HBP), capable of binding either the parasite or EF. Leishmania may thus be attached initially to the macrophage via such a galactose-binding site and be subsequently engulfed by the macrophage.

For attachment study of promastigotes to macrophages, the culture system of Hamman & Spiro (7) was adapted. Peritoneal exudate cells (PEC), mostly macrophages, from C3H mice were harvested 5 days after I.P. stimulation with 2 ml thioglycolate. On the day of harvest, the cells were collected in McCoy's medium containing 5 units of heparin, 100 μg streptomycin and 100 units penicillin per ml. The cell concentration was brought to 5 × 10⁷ cells/ml and 1 ml was plated into each well of the 24 wells microplate. Before adding the PEC, 12 mm diameter sterile coverslips were placed in each well. 24 hours after incubation at 37°C in an atmosphere of 5% CO₂ in air, the medium was changed, removed, and 200 μl of the sugar in the desired concentration made in Hank's balanced salt solution containing 1% albumin (na55h) was added. After 15 min at 37°C, the sugar was replaced with 400 ml
of the same sugar, at the same concentration containing $2 \times 10^7$ washed promastigotes. After a further 10 min at $37^\circ C$, the PEC were washed 5 times with phosphate buffer, fixed and stained with Giemsa. Following counting, the parasite attachment index (PAI), ($\S 10$) was calculated.

Figure 4 shows that different sugars (at $0.3 - 0.5$ M) inhibit the attachment of promastigotes to macrophages. Lactose, Gal-$\beta$(1→4)Glc, being the most efficient. Lower concentrations of sugars promote attachment while $1$ M and higher concentrations are toxic to host (macrophage) cells. Apart from standard error possible (Eq. 10C), the effect of lower saccharide concentrations may be due to additional sugar nutrient. Sugars at concentrations of $0.5$ M are non-toxic to both promastigotes and macrophages. Following the addition of $0.5$ M saccharide and incubation as described, the cells are washed and give normal infective rates.

6. Conclusions

In our first report ($\S 7$) we have proposed that surface carbohydrate may play a role in the binding of Leishmania promastigotes to macrophages. Our current experiments ($\S 5$) support this hypothesis. Thus, Leishmania, an intracellular obligatory parasite, appears to use a cellular mechanism designed inter alia to remove damaged cells from blood circulation (6) as a vehicle to enter host cells. Once inside, the LF produced has a protective function, being inhibitory to the host’s lysosomal enzymes (8).

Our analytical results ($\S 3$) already suggest galacto derivatives as possible biosynthetic inhibitors of LF. As soon as more advanced structural results are available and larger structural elements become known, and in view of our original research proposal, one will be in a better position as far as the design of such inhibitors is concerned.

A most important outcome of our work is a specific radioimmunoassay for Leishmania ($\S 4c$). Effort is to be dedicated to the standardization of the method – to make it diagnostically useful. Additionally, the scope of the method should be widened to more acute types of Leishmania.

7. Proposals for Further Research (detailed proposal submitted along with this report)

(i) Purification and structural work on EF. Here we will be employing the affinity chromatography purification ($\S 2i$).

(ii) Radioimmunoassay (RIA) for Leishmania. Standardization of the method. Application to human and animal cases.

(iii) Biological role of EF. Use of labeled EF ($\S 4a$ and $4b$) to localize EF. Role of LF in “conditioning”.

(iv) Immunogenicity of EF. Use of MDP, mycolic acid etc. to make LF immunogenic.

(v) Inhibitors. Based on structural features of LF, inhibitors to its biosynthesis will be designed.
Fig. 1  The effect of different concentrations of coating *L. tropica* EF on the binding of antibodies from homologous antiserum at dilution 1:25. The EF showed a precipitating line with whole antiserum at 1000 μg/ml.
Fig. 2  Binding of □ rabbit anti *L. tropica* antiserum, □ rabbit anti *L. donovani* antiserum and ■ normal rabbit serum to plates coated with *L. donovani* or *L. tropica* EP.

SERUM DILUTIONS
Fig. 3  
Effect of absorption of anti *L. tropica* and anti *L. donovani* antiserum with homologous and heterologous EF. ○ - Anti *L. tropica* absorbed to homologous EF; ▲ - Anti *L. donovani* absorbed to homologous EF. The inhibition is calculated by dividing the antiserum absorbed to homologous EF by the same antiserum absorbed to heterologous EF.
Fig. 4 The effect of sugars at different concentrations on the parasite attachment index (PAI) of *L. tropica* (L 137) to C3H mouse macrophages.

- Lactose; ○ - Glucose; ● - Methyl α-D-galactopyranoside;
- Methyl β-D-galactopyranoside; ▲ - Raffinose; △ - Methyl-α-D-mannopyranoside; ▼ - D-arabinose
9. Literature Cited


10. Glossary

EF Factor excreted by *Leishmania* (Excreted Factor). EF preparations are designated with digits indicating the leishmanial source.

**Leishmanial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L137</td>
<td><em>Leishmania tropica</em></td>
<td>LRC L137</td>
</tr>
<tr>
<td>L52</td>
<td><em>Leishmania donovani</em></td>
<td>LRC L52</td>
</tr>
</tbody>
</table>

These strains were obtained from the WHO *Leishmania* Reference Centre collection maintained in the Department of Protozoology in Jerusalem.

PNA peanut lectin

RIA radioimmunoassay

PAI parasite attachment index =

\[
\frac{a \exp x b \exp x 100}{a \text{cont} x b \text{cont}}
\]

a = percent of host cells where attachment is apparent. 400 host cells were counted.

b = average no. of attached promastigotes per cell. 100 cells with attached promastigotes were counted.

(i) Figures related to attachment represent a proportion of already engulfed promastigotes.

(ii) Saccharides were included in experiments and avoided in controls.

11. Distribution List

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