IVERMECTIN, LEVAMISOLE AND THYMIC EXTRACT FOR CHEMOTHERAPY AND IMMUNOSTIMULATION OF VISCERAL LEISHMANIASIS IN HAMSTERS AND MICE

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SUMMARY: The anti-leishmanial activity of ivermectin, pentostam or combination of pentostam with either levamisole or thymic extract was tested against Leishmania donovani infection in hamsters and mice. In vitro peritoneal macrophage-parasite interaction, the effect of splenic cells on the interaction of macrophages and parasites, spleen weight, parasite burden in spleen tissue as well as the antibody titers using micro-ELISA were used as parameters for evaluating the efficacy of these chemotherapeutic regimens. Treatment with ivermectin and immunoenhancement with pentostam combined with levamisole gave best results in both animal models. Furthermore, regimens used in this work were all active in reducing phagocytosis of promastigotes by macrophages in vitro.

INTRODUCTION

The causative agent of visceral leishmaniasis, Leishmania donovani, produces morbidity and mortality in many areas of the world. The flagellated protozoan agent parasitizes tissue macrophages wherein the agent resides as an obligate parasite (1,2). Disseminated leishmaniasis may be difficult to cure with chemotherapeutic agents, as is the case with other clinical forms of human leishmaniasis. To date, no effective nontoxic therapy for L. donovani infections has been available (3,4). Chemotherapeutic agents in use include several pentavalent...
antimonials, amphotericin and purine analogs (5-7). These drugs when used as indicated may exert severe toxic reactions in treated individuals. Furthermore, variability in response to therapy may be due to parasitologic factors such as sensitivity of the organisms to antimony, pharmacologic factors such as absorption and perfusion of the drug to infected sites, and immunologic factors such as ability to mount cellular or humoral responses to the parasite (8). When drug treatment of this and other recalcitrant infections is successful, however, the cooperation of the immune mechanisms of the host is required.

In human visceral leishmaniasis, no effective nor sustained state of cell-mediated immunity is achieved (9). Thus, the infection in untreated cases proceeds until enormous parasite burdens are present in the liver and spleen. This represents an immunological deficit which probably has a genetic basis (10,11). Experimental evidence showed that immunological deficit can be overcome by appropriate stimulation of the macrophage system leading to sufficient killing of *L. donovani* amastigotes (12,13). Furthermore, immunostimulation resulting in macrophage activation combined with chemotherapy was shown to provide a therapeutic regimen superior to either one alone (6,14).

The strategies which are now exploited in the treatment of leishmaniasis include specific or nonspecific immunostimulation with a number of immunoenhancing agents such as microbial products, glucan polymers, cyclosporine A and monoclonal anti-Ia antibodies (6,15-20). These studies documented the effectiveness of immunostimulation in reducing the parasite burden in *L. donovani* infected mice.

Various reports indicated that levamisole and thymic extract give a more favorable influence, in comparison with other immunoenhancing agents, on the immunological response of mammals and humans (21,22). Levamisole was found to stimulate healing of cutaneous lesions due to *L. tropica* in mice (23). On the other hand, ivermectin (22, 23-dihydroderivative of a B1, a macro-cyclic lactone), a broad spectrum anti-parasitic agent, has become a promising drug for treating parasitic infections (24,25). The objective of this study was to test, in in vitro and in vivo models in hamsters and mice, the efficacy of pentostam in combination with levamisole and thymic extract as immunostimulating agents. In addition, ivermectin was included in this study to investigate its anti-leishmanial activity.
MATERIALS AND METHODS

Parasite and experimental host:  L. donovani (MHOM/IQ/1982/BRC1 strain) being maintained in our laboratory by continual passages in Balb/C mice. Suspensions of parasites for inoculation into experimental animals were prepared from promastigotes cultured in biphasic modified NNN medium (26).

Male outbred golden hamsters (Mesocricetus auratus) and Balb/C mice from our colony were kept in air-conditioned quarters with food and water provided ad libitum.

Infection: Experimental animals were infected by intracardial injection of 1 × 10^7 promastigotes.

Treatment protocol: Treatment was started one month postinfection and continued for 10 days. Fifteen hamsters and 30 mice were weighed and divided into five groups (groups No. g1 - g5 for hamsters, and g6 - g10 for mice). Drugs were administered intramuscularly once a day. Treatment protocol was as follows:

1- g1 and g6 were treated with ivermectin (Merck and Sharp, England) at a dose of 0.3 mg/100 g body weight (b.w.)/day (24).

2- g2 and g7 were treated with pentostam (sodium stibogluconate, Wellcome Foundation Ltd., England) at a dose of 2 mg/100 g b.w./day (6).

3- g3 and g8 were treated with levamisole (Merck and Sharp) at a drug dose level of 0.25 mg/100 g b.w./day (27) plus pentostam 2 mg/100 g b.w./day.

4- g4 and g9 received bovine thymic extract (28) at a dose of 1 mg/100 g b.w./day (29) plus pentostam 2 mg/100 g b.w./day.

5- g5 and g10 were injected with phosphate buffered saline (PBS) and served as control groups.

Two weeks after the last injection, animals in each treatment group were weighed, sampled for blood and then killed with ether vapor. Spleen weight was recorded, and impression slides were made from the spleen tissue. The slides were fixed with methanol, stained with giemsa stain and the parasite burdens were estimated by the method of Stauber (30). Pieces of the spleen and liver were cultured on NNN medium for detection of L. donovani promastigotes.

In vitro macrophage-parasite interaction: Interaction between peritoneal macrophages from treated and control animals infected with L. donovani and the parasites was evaluated in vitro, as reported by Cook et al (31). Data were expressed by the following equation:

\[
\text{Number of amastigotes/100 macrophages (experimental cultures)} \times 100 \\
\text{Number of amastigotes/100 macrophages (control cultures)}
\]
Effects of spleen cells on the in vitro macrophage-parasite interaction: Spleen cells from treated and control animals infected with \textit{L. donovani} (1.4 \times 10^5 cells/ml) were used. Macrophages were obtained from normal animals having received a single ip injection of a 5\% glycogen solution 3 days before harvesting peritoneal exudate cells. Spleen cells were mixed with promastigotes and it was added to the macrophage monolayers.

Enzyme linked immunosorbent assay (ELISA): Sera from treated and control animals were tested for anti-\textit{L. donovani} antibodies by the micro-ELISA technique (32).

Statistical analysis: The student ¨t¨ test was used to test the significance in the different groups.

RESULTS

\textit{In Vitro Macrophage-parasite Interaction}

The percent leishmanial infectivity of macrophages obtained from control noninfected and nontreated animals was 77\% for hamsters and 87\% for mice. The percentages of phagocytosis by macrophages taken from different experimental

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Percentage of phagocytosis (mean ± SEM)</th>
<th>Group No.</th>
<th>Percentage of phagocytosis (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g1</td>
<td>39.7 ± 2.6\textsuperscript{a}</td>
<td>g6</td>
<td>32.9 ± 2.3\textsuperscript{a}</td>
</tr>
<tr>
<td>g2</td>
<td>41.7 ± 6.4\textsuperscript{b}</td>
<td>g7</td>
<td>37.1 ± 2.4\textsuperscript{a}</td>
</tr>
<tr>
<td>g3</td>
<td>47.3 ± 1.5\textsuperscript{c}</td>
<td>g8</td>
<td>31.5 ± 2.4\textsuperscript{a}</td>
</tr>
<tr>
<td>g4</td>
<td>38.7 ± 3.9\textsuperscript{d}</td>
<td>g9</td>
<td>32.6 ± 3.6\textsuperscript{a}</td>
</tr>
<tr>
<td>g5</td>
<td>68.7 ± 0.9</td>
<td>g10</td>
<td>70.2 ± 1.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}: P<0.001 (g1 versus g5 and g6-g9 versus g10)
\textsuperscript{b}: P<0.02 (g2 versus g5)
\textsuperscript{c}: P<0.01 (g3 versus g5)
\textsuperscript{d}: P<0.002 (g4 versus g5).
animal groups are listed in Table I. Pentostam and pentostam in combination with levamisole or thymic extract were equally effective in this model in both hamsters and mice. These results indicate that no increase in activity was obtained from that of pentostam alone. Ivermectin was also effective in activating macrophages when given to infected animals, resulting in lower percent of phagocytosis in the in vitro model.

Effect of Spleen Cells on the in vitro Macrophage-parasite interaction

The effects of intact splenic cells from infected and treated and infected and nontreated animals on the interaction between _L. donovani_ promastigotes and peritoneal macrophages from normal animals are presented in Table II. In this

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Percentage of infected macrophages after addition of splenic cells (mean ± SEM)</th>
<th>Suppression index&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>g1</td>
<td>51.29 ± 3.10</td>
<td>42.48</td>
</tr>
<tr>
<td>g2</td>
<td>54.11 ± 4.18</td>
<td>39.32</td>
</tr>
<tr>
<td>g3</td>
<td>61.47 ± 1.92</td>
<td>31.06</td>
</tr>
<tr>
<td>g4</td>
<td>50.21 ± 2.70</td>
<td>43.69</td>
</tr>
<tr>
<td>g5</td>
<td>89.17 ± 2.50</td>
<td>0.00</td>
</tr>
<tr>
<td>g6</td>
<td>41.59 ± 2.29</td>
<td>53.16</td>
</tr>
<tr>
<td>g7</td>
<td>47.01 ± 2.16</td>
<td>47.07</td>
</tr>
<tr>
<td>g8</td>
<td>39.87 ± 2.09</td>
<td>55.10</td>
</tr>
<tr>
<td>g9</td>
<td>41.22 ± 2.54</td>
<td>53.58</td>
</tr>
<tr>
<td>g10</td>
<td>88.81 ± 1.55</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> Suppression index =

\[
\frac{\text{Percentage of infected macrophages from infected and treated animals}}{\text{Percentage of infected macrophages from infected and nontreated controls}} \times 100
\]
model, splenic cells from infected animals treated with different chemotherapeutic agents were able to significantly reduce the parasite – macrophage interaction as indicated by the lower percent of infected cells. The index of suppression of the interaction was high when ivermectin and combination of pentostam with thymic extract were used for treating hamsters. In mice, ivermectin and combination of pentostam and levamisole or thymic extract resulted in reducing phagocytosis when compared with that due to pentostam alone.

Effect of Chemotherapy on Spleen Weight and Parasite Burden

The effects of different chemotherapeutic agents on spleen weight and on parasite burden are presented in Table III. Hamsters administered pentostam alone after infection resulted in great increase in spleen weight when compared with that of infected nontreated group. The use of either levamisole or thymic extract in combination with pentostam was effective in reducing spleen weight as compared with pentostam alone. In Balb/C mice, only pentostam alone or the combination with levamisole were associated with significantly lower spleen weight.

In respect of parasite burden depicted in Table III, ivermectin, pentostam and pentostam combined with levamisole resulted in reducing parasite burden of hamsters as compared with infected nontreated controls. Furthermore, pentostam given with levamisole caused decreased infection levels as compared with the total spleen parasite burden in pentostam-treated hamsters. Thymic extract-pentostam treated hamsters exhibited an approximately twofold increase in spleen parasite burdens as compared with pentostam-treated animals. Furthermore, ivermectin treatment reduced parasite burden by 89.17% as compared with nontreated hamsters. In mice, pentostam in a dose of 2 mg/100 g was not effective in reducing total parasite burden (Table III). In contrast, combination of pentostam and levamisole or thymic extract resulted in reducing parasite burden. Treatment of infected mice with ivermectin resulted in 75.88% reduction of parasite burden as compared with control nontreated animals. Moreover, ivermectin treatment demonstrated approximately a three-fold decrease in amastigote number when compared with pentostam-treated mice.

Detection of Antibodies to L. donovani in Treated Animals

In the present study, screening by ELISA for antibodies in sera from treated hamsters and mice showed lower levels of antibodies against L. donovani when compared with the infected and nontreated control group (Table IV). Treatment
Table III. Spleen weight and parasite burden of hamsters and mice infected with *L. donovani* and treated with different chemotherapeutic agents

| Group No. | Spleen weight (mg) (mean ± SEM) | Number of amastigotes/spleen (mean ± SEM ×10⁷) | Percentage of decrease in amastigote number
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>g1</td>
<td>240.0 ± 69.3</td>
<td>3.97 ± 1.01</td>
<td>89.17</td>
</tr>
<tr>
<td>g2</td>
<td>533.3 ± 55.1</td>
<td>12.63 ± 6.23</td>
<td>65.56</td>
</tr>
<tr>
<td>g3</td>
<td>276.7 ± 101.7</td>
<td>6.40 ± 2.46</td>
<td>82.55</td>
</tr>
<tr>
<td>g4</td>
<td>280.0 ± 68.1</td>
<td>25.83 ± 10.83</td>
<td>29.56</td>
</tr>
<tr>
<td>g5</td>
<td>316.7 ± 86.5</td>
<td>36.67 ± 20.67</td>
<td>0.00</td>
</tr>
<tr>
<td>g6</td>
<td>214.3 ± 24.3</td>
<td>2.06 ± 0.62</td>
<td>75.88</td>
</tr>
<tr>
<td>g7</td>
<td>150.0 ± 18.8b</td>
<td>6.68 ± 3.27</td>
<td>21.78</td>
</tr>
<tr>
<td>g8</td>
<td>138.8 ± 10.3c</td>
<td>3.43 ± 2.04</td>
<td>59.83</td>
</tr>
<tr>
<td>g9</td>
<td>286.7 ± 33.0</td>
<td>2.68 ± 1.28</td>
<td>68.62</td>
</tr>
<tr>
<td>g10</td>
<td>263.3 ± 26.0</td>
<td>8.54 ± 7.21</td>
<td>0.00</td>
</tr>
</tbody>
</table>

a: Percentage of decrease in amastigote number =

\[
100 - \left( \frac{\text{Number of amastigotes/ spleen of infected and treated animals}}{\text{Number of amastigotes/ spleen of infected and nontreated controls}} \times 100 \right)
\]

b: \( P < 0.01 \) (g7 versus g10)

c: \( P < 0.001 \) (g8 versus g10).

with ivermectin or pentostam in combination with levamisole in hamsters resulted in the most substantially decreased antibody titers. The use of thymic extract with pentostam was not superior to pentostam alone as judged by insignificant differences in antibody titers.

The major results of the present study are: [1] ivermectin was more effective than pentostam against *L. donovani* both in in vivo and in vitro assays in both hamsters and mice. [2] Combination of levamisole or thymic extract with pentostam was more active than pentostam alone mainly in mice in this model.
Table IV. Effects of different treatment regimens on anti-\textit{L. donovani} antibodies measured by ELISA in hamsters and mice

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Antibody titer$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>g1</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>g2</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>g3</td>
<td>0.41 ± 0.10</td>
</tr>
<tr>
<td>g4</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td>g5</td>
<td>0.62 ± 0.08</td>
</tr>
<tr>
<td>g6</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>g7</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>g8</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>g9</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>g10</td>
<td>0.35 ± 0.01</td>
</tr>
</tbody>
</table>

$^a$: Each figure represents the absorbance at 490 nm.

**DISCUSSION**

Chemotherapeutic treatment of leishmaniasis requires intense and prolonged regimens with overtly toxic drugs (33). Therefore, any alternative or auxiliary therapeutic measures which allow reduction in the dosage or replacement of antileishmanial drugs required to achieve cure would be valuable. Ivermectin, a broad spectrum anti-parasitic drug, successfully used by Yousif et al. (24) for treating mange, was assayed in the present study for any anti-leishmanial activity by in vivo and in vitro experiments. Furthermore, in virtually all types of infection, a brisk and appropriate immune response by the infected host augments significantly the effectiveness of chemotherapeutic drugs. Thus, a prime candidate for auxiliary therapy in a variety of chronic protozoal and bacterial infections is immunoenhancement (15). Before such combined therapy can be considered for trials in man, however, appropriate experimental models need to be explored thoroughly.
Our experiments provide evidence of enhanced nonspecific resistance of hamsters and mice against visceral leishmaniasis. Ivermectin as well as combination of levamisole or thymic extract with pentostam administration markedly attenuated the course of L. donovani infection, as indicated by reduced proliferation of amastigotes in spleen tissues. Peritoneal macrophages obtained from infected and treated hamsters and mice also exhibited a reduced capacity of phagocytosis of the parasites in vitro. The mechanism(s) whereby these agents reduce the percentage of infected macrophages in vivo and in vitro remain to be delineated. The accumulated evidences from studies on isolated cells, on experimental animals, on healthy volunteers and on patients strongly suggest that levamisole and thymic extract restore the function of phagocytes and T lymphocytes from compromised hosts to normal (34,35). However, the results presented herein suggest that ivermectin, levamisole and thymic extract have exerted their effects by several mechanisms. It is quite possible that these agents have adverse effects on the intracellular growth of amastigotes or stimulate macrophages by increasing their ability to kill the amastigote form of the parasite as was the case with cyclosporine-A and its analog B-5-49 (19). Treatment with these agents could increase the proportion of cytotoxic T-lymphocytes in the total lymphocyte population in the spleen as was the case with levamisole and viral myocarditis due to Coxsackie virus (34). Furthermore, experiments to enhance therapeutically the depressed delayed-type hyper-sensitivity response seen during visceral leishmaniasis were successful by treatment regimens reported here (data not shown).

Purified granulocyte-macrophage colony-stimulating factor is potentiated in the ability to eradicate L. tropica in vitro by mouse peritoneal macrophages (36). Treatment of mice with glucan (an immunopotentiating agent) augments release of such factors from macrophages (37). Haidaris and Bonventre (12) observed that elimination of L. donovani amastigotes by activated mouse macrophages is lymphokine dependent. Macrophages activated by either Corynebacterium parvum or Mycobacterium tuberculosis are ineffective in killing amastigotes unless the activated state is maintained by daily addition of lymphokine to infected monolayers (37). Thus, chemotherapy and immunonormalization as examined in the present study might help the continuous presence of lymphokine from splenic cells both in vivo and in vitro necessary for elimination of amastigotes by macrophages.

Levamisole and thymic extract may be more advantageous than certain bacterial immunostimulating agents such as BCG and C. parvum. The microbial
agents have several disadvantages relating to their bacterial nature, antigenic qualities, difficulty of standardization, possible infectious complications, unknown metabolites and the fact that the immunostimulant is, in most cases, an unknown entity (38). On the contrary, levamisole and thymosine are chemically defined and in clinical trials appear to be safe (17,18).

REFERENCES