Activity of Amphotericin B Cholesterol Dispersion (Amphocil) in Experimental Visceral Leishmaniasis

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Standard therapy of human visceral leishmaniasis with parenteral pentavalent antimonial agents is generally curative but has the disadvantages of a 28-day treatment course, occasional treatment failures, and toxicity. The antifungal and antileishmanial agent amphotericin B has been complexed with lipids to develop a less toxic formulation of amphotericin B. Because lipid particles are phagocytized by the reticuloendothelial system, lipid-associated amphotericin B should be concentrated in infected macrophages and be very effective against visceral leishmaniasis. One formulation, amphotericin B cholesterol dispersion (ABCD) (Amphocil), was tested for antileishmanial activity in Leishmania donovani-infected hamsters. In the first experiment, hamsters were infected, administered with the drug 3 days later, and then sacrificed after a further 4 days. ABCD (dose needed to suppress 99% of hepatic parasites compared with controls [SD (99)], 0.4 mg/kg of body weight) was 15 times as effective as conventional amphotericin B (SD (99), 6.0 mg/kg). Pentavalent antimony in the form of meglumine antimonate had an SD (84) of 416 mg/kg. In a second experiment in which animals were allowed to become more heavily infected, the drug was administered 10 days after infection and the animals were sacrificed after a further 2, 7, or 11 days. ABCD was approximately four times as active as conventional amphotericin B. These experiments suggest that ABCD is at least four times as active as conventional amphotericin B against visceral leishmaniasis and that clinical trials are warranted.

Leishmaniasis results from infection with protozoal parasites of the genus Leishmania. Insect-transmitted forms of leishmaniasia are inoculated into the skin via the bite of a female sandfly. The insect-transmitted forms cannot survive long in a mammalian host: within a few hours, the only parasites to be seen are within mononuclear phagocytes. Throughout the rest of the interaction between the parasite and its human host, leishmaniasia are obligate intramacrophage microorganisms. Visceral leishmaniasis results from infection of the macrophages of the liver, spleen, and bone marrow with leishmania. The symptoms of established disease are fever, weight loss, hepatosplenomegaly, and pancytopenia. If untreated, established disease is characteristically fatal because of intercurrent infections such as diarrhea and pneumonia.

The first antileishmanial chemotherapeutic agents with a favorable therapeutic index, the pentavalent antimonial agents (Sb), were introduced in the 1940s and are still the primary therapy for all forms of leishmaniasia. The formulations available then and now are sodium stibogluconate (Pentostam), in which Sb is reacted with gluconic acid to form an unknown number of compounds of unknown structure (3), and meglumine antimonate (Glucantime), in which pentavalent antimony is reacted with the sugar meglumine to form a similarly unknown set of products. Although Sb will heal approximately 90% of patients with visceral disease (10), there are several disadvantages to Sb therapy: the standard regimen is 28 days (40 days in India) of parenteral injections, modest to intolerable toxicity occurs, and a small percentage of cases (~10%) are Sb treatment failures.

Conventional amphotericin B desoxycholate is an effective agent for treatment of the leishmaniasias but is little employed because of the side reactions of fever and phlebitis during infusion, anemia, kidney dysfunction, and hypokalemia. The reason that this antifungal agent is also an anti-leishmanial agent is that both leishmania and fungi contain a 24-substituted sterol (ergosterol or episterol) as the major membrane sterol (2, 9), whereas in mammalian cells the major sterol is cholesterol. Amphotericin B preferentially interacts with 24-substituted sterols; toxicity is a consequence of binding of amphotericin B to host cell cholesterol. The clinical demand for less toxic antymycotic agents is such that there is an intense effort to decrease the toxicity of amphotericin B by encapsulating the drug within lipid. The purpose of lipid encapsulation of amphotericin B is to generate a particle which, relative to desoxycholate micelles, has a decreased tendency to release amphotericin B during infusion, a decreased tendency to be distributed to the kidney, and an increased tendency to provide the drug to infecting fungi.

Lipid-encapsulated particles are primarily removed from the circulation by mononuclear phagocytes. Formulation of amphotericin B with lipid therefore results in a complex in which a biochemically rational, clinically effective antileishmanial agent is designed, albeit adventitiously, to specifically enter the cells in which leishmaniasia reside. One of three clinical formulations of lipid associated amphotericin B is amphotericin B cholesterol dispersion (ABCD) (Amphocil). ABCD consists of cholesteryl sulfate and amphotericin B (1:1 molar ratio) in disks of about 100 nm in diameter and 4 nm in width (1). In preparation for clinical testing of ABCD against human kala-azar, we determined the relative efficacies of ABCD and conventional amphotericin B in viscerally infected hamsters.

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TABLE 1. Efficacies of ABCD, conventional amphotericin B, and meglumine antimonate in hamsters infected with *L. donovani* for 3 days prior to drug administrationa

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dose (mg/kg)</th>
<th>No. (106) parasites/ liver</th>
<th>% Suppression of parasitesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCD</td>
<td>0.1</td>
<td>85</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>10</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Conventional amphotericin B</td>
<td>0.1</td>
<td>661</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>184</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>37</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>12</td>
<td>99</td>
</tr>
<tr>
<td>Meglumine antimonate</td>
<td>52</td>
<td>717</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>321</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>416</td>
<td>165</td>
<td>84</td>
</tr>
<tr>
<td>5% Dextrose in water</td>
<td>1,031</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

a Animals were infected with *L. donovani*. Drug was administered 3 days later, and animals were sacrificed 4 days after dosing.
b Mean no. of parasites per liver for seven to eight animals per group.

TABLE 2. Efficacy of ABCD or conventional amphotericin B in hamsters infected with *L. donovani* for 10 days prior to drug administrationa

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dose (mg/kg)</th>
<th>% Suppression when animals were sacrificed on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>ABCD</td>
<td>0.1</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>97</td>
</tr>
<tr>
<td>Conventional amphotericin B</td>
<td>0.1</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>70</td>
</tr>
<tr>
<td>5% Dextrose in water</td>
<td>10</td>
<td>(97)</td>
</tr>
</tbody>
</table>

a Animals were infected with *L. donovani*. Drug was administered 10 days later. The animals were sacrificed 2, 7, or 11 days after dosing.

MATERIALS AND METHODS

Hamster experiments. Hamsters were infected with *Leishmania donovani* (WR# 378) and administered with drugs exactly as described previously (4, 8). In brief, 60- to 80-g hamsters (*Mesocricetus auratus*) were administered intracardiac injections of approximately 10⁷ *L. donovani* amastigotes (Khartoum strain). Three days later (lightly infected animals) or 10 days later (heavily infected animals), the six to eight animals in each group were administered with 5% dextrose in water (negative controls), conventional amphotericin B, ABCD, or meglumine antimonate (positive controls). All preparations were administered via intracardiac injection in less than 1 min, except for meglumine antimonate, which was given intramuscularly. The animals were sacrificed 2 to 14 days after the single injection of the drug, and the number of parasites per liver was determined from the ratio of parasites per liver cell nucleus and from the weight of the liver, as previously described (8).

Computations. For each experimental group, the mean number of parasites per liver was determined from the values for the individual animals, and percent parasite suppression was calculated as follows: [(mean number of parasites in negative controls) – (mean number of parasites in the experimental group)]/(mean number of parasites in negative controls) x 100.

Drugs. ABCD was supplied by Liposome Technology, Inc., Menlo Park, Calif. The formulation contains cholesteryl sulfate and amphotericin B in a molar ratio of 1:1. Amphotericin B desoxycholate (conventional amphotericin B) and meglumine antimonate were purchased from Squibb, Princeton, N.J., and Rhone Poulenc, Paris, France, respectively. The quantity of administered drugs refers to the amount of active agent (amphotericin B or Sb) administered.

RESULTS

Lightly infected hamsters. The results of administering ABCD, conventional amphotericin B, or meglumine antimonate to hamsters infected with *L. donovani* for 3 days are shown in Table 1. Four days after the single injection of drug, and a total of 7 days after infection with parasites, there were approximately 10⁶ organisms per liver. Under these conditions, the dose of meglumine antimonate that is expected to suppress 50% of parasites (SD 50) was between 52 and 104 mg of Sb per kg of body weight, and the SD (90) was more than 416 mg of Sb per kg. For comparison, the human curative dose, which may be considered an SD (99%), is 20 mg of Sb per kg/day for 28 days (560 mg of Sb per kg). Conventional amphotericin B was a much more effective agent than meglumine antimonate on a milligram per kilogram basis. The SD (50) was between 0.1 and 0.4 mg/kg, and the SD (90) was between 0.4 and 1.5 mg/kg. A dose level of 6.0 mg of conventional amphotericin B per kg suppressed 99% of parasites. Amphotericin B in the form of ABCD was more active than conventional amphotericin B. The SD (50) and the SD (90) were less than the lowest dose tested (0.1 mg/kg), and the SD (99) was 0.4 mg/kg. On the basis of comparative SD (99%), ABCD was 15 times as active as conventional amphotericin B.

Heavily infected hamsters. The results of administering ABCD and conventional amphotericin B to hamsters infected with *L. donovani* for 10 days are shown in Table 2. This second experiment was designed to investigate whether the relative activity of ABCD versus conventional amphotericin B was maintained in heavily infected hamsters and to investigate the length of time that either formulation would be active after one administration. Thus, the animals were allowed to become more heavily infected prior to drug administration, and the animals were sacrificed at different intervals after dosing.

In animals administered with dextrose and sacrificed 2 days later (12 days after infection with parasites), there were more organisms (1.7 × 10⁶ per liver) than in the lightly infected animals in experiment 1. Organisms in control animals approximately doubled by 5 days later (17 days after infection) and approximately tripled after a further 4 days (21 days after infection). If we assume that the animals were reproducibly infected with *L. donovani*, then the doubling time of *L. donovani* in this model is approximately 4 days.

At each of the three times after infection on which the animals were sacrificed, suppression by 0.4 mg of ABCD per day was reproducible.
kg was approximately equal to suppression by 1.5 mg of conventional amphotericin B per kg. Thus, in heavily infected hamsters, ABCD was approximately 4 times as active as conventional amphotericin B.

For most drug doses, there was more parasite suppression 7 days after drug administration than 2 days after drug administration. Increased suppression occurred for both ABCD- and conventional amphotericin B-treated animals. When increased suppression occurred, the increases were modest and in the range of 15 to 25%. There was no further increase in suppression between 7 and 11 days after drug administration. The increase in suppression between 2 and 7 days may mean that residual amphotericin B remains in the liver during that time and eliminates ~20% more parasites. It is also possible that the physical disappearance of dead parasites requires more than 2 days and that the apparent decrease in parasite numbers between days 2 and 7 simply reflects the time required for digestion of dead leishmaniae.

**DISCUSSION**

Amphotericin B is insoluble in water. The conventional clinical preparation is in reality a deoxycholate micellar suspension of the drug. The attempt to improve upon the therapeutic index of amphotericin B deoxycholate by formulating lipid-associated amphotericin B preparations thus results in replacing an old lipid-associated drug formulation with new lipid-associated drug formulations. Three new preparations of lipid-associated amphotericin B are now in clinical trial as antymycotic or antileishmanial agents: ABCD; amphotericin B encapsulated in liposomes containing phosphatidylcholine, cholesterol, and distearoylphosphatidylglycerol (Ambisome [Vestar]); and amphotericin B complexed with dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol (ABLC [Bristol Myers Squibb]).

This study demonstrates that in hamsters viscerally infected with *L. donovani*, amphotericin B in the form of ABCD is 15 times (lightly infected animals) or 4 times (heavily infected animals) as active as amphotericin B as a deoxycholate micelle. Although the improvement in efficacy in these experiments is greater than the improvement when amphotericin B in the form of ABLC was used in this model (4) or when amphotericin B in the form of distearoylphosphatidylglycerol was used in a mouse model (5), the experiments were performed at different times or in different models and the results are noncomparable.

The improvement in hepatic antileishmanial efficacy of ABCD demonstrated here is consistent with the increased removal of ABCD, compared with conventional amphotericin B, from the circulation by the liver and bone marrow. In preclinical work, dogs were administered with 0.6 mg of ABCD or conventional amphotericin B per kg. Levels of ABCD in plasma were one-fifth the levels of amphotericin B deoxycholate, levels of ABCD in liver and bone marrow were 1.7 to 2.7 times higher than those of amphotericin B deoxycholate, and levels of ABCD in kidneys were one-seventh the levels of amphotericin B deoxycholate (7).

The hamster efficacy experiments and the dog drug distribution data suggest that ABCD should be two to four times more effective than conventional amphotericin B against visceral leishmaniasis. The drug distribution study also suggests that ABCD should be less toxic to kidneys than conventional amphotericin B. If these suggestions hold for the clinical situation, the total amount of amphotericin B normally administered to patients with leishmaniasis could be cut at least in half, and the drug could be given in relatively large daily doses. The relatively slight increase in antileishmanial activity between 2 and 7 days after drug administration suggests that the dosing interval should be closer to 2 days than to 7 days. The amount of conventional amphotericin B normally required to cure human visceral leishmaniasis is not well investigated but is probably not more than 30 mg/kg. Davidson et al. have reported the successful treatment of one patient administered with 1 mg of amphotericin B encapsulated in phosphatidylcholine, cholesterol, and distearoylphosphatidylglycerol per kg per day for 21 days (6). Clinical trials of higher daily doses of ABCD administered over a shorter period of time are now in progress.

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