Hexadecylphosphocholine (miltefosine), a membrane-active alkylphospholipid, may be the first effective oral agent for visceral leishmaniasis, an intracellular protozoal infection of tissue macrophages. In vitro, miltefosine stimulates T cells and macrophages to respond to and secrete activating cytokines, including interferon (IFN)-γ, and enhances macrophage production of microbicidal reactive nitrogen and oxygen intermediates (RNIs and ROIs, respectively). To determine whether these effects mediate miltefosine's in vivo leishmanicidal efficacy, genetically deficient mice were infected with Leishmania donovani. Intracellular visceral killing was retained in mice lacking or deficient in T cells, endogenous IFN-γ, and macrophage generation of leishmanicidal RNIs and ROIs. Although mutant mice responded to miltefosine in the absence of tissue granulomas, treatment enhanced granuloma assembly in normal animals. These results suggest that miltefosine's visceral leishmanicidal effect does not require host T cell-dependent or activated macrophage-mediated mechanisms; thus, this agent may potentially be useful in treating T cell-deficient patients with kala-azar.

Hexadecylphosphocholine (miltefosine) is a membrane-active alkylphosphocholine that was originally developed as an oral antineoplastic agent. However, miltefosine also exerts experimental effects against leishmania, pathogenic protozoans that replicate in tissue macrophages, and is particularly active in models of visceral leishmaniasis (kala-azar) caused by infection with intracellular Leishmania donovani and L. infantum [1–3]. These findings, together with safety data derived from original studies in cancer patients, led to recent clinical trials in Indian kala-azar. The results of these currently ongoing studies suggest that miltefosine may be the first successful oral therapy for visceral leishmaniasis [4].

Miltefosine is directly toxic to the promastigote form of leishmania [1], which develops in the sandfly vector. However, its action in the tissues against intracellular amastigotes (to which promastigotes transform) may be more complex and may involve effects on the macrophage itself or on activating T cell-dependent responses. For example, by utilizing mononuclear cells including T cells and macrophages, the following in vitro actions have been ascribed to miltefosine: priming for or costimulating cytokine-related events, including mRNA expression for interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor; secretion of IFN-γ and tumor necrosis factor-α; expression of surface cytokine receptors and major histocompatibility complex class II molecules (HLA-DR) [5–8]; and inducing or enhancing respiratory burst activity and nitric oxide (NO) release [6–8], the macrophage’s primary microbicidal mechanisms [9, 10].

Coupled with the general immunomodulatory actions of membrane-stimulating alkylphospholipids, the preceding effects raise the possibility that miltefosine-induced immunoenhancement may contribute to its leishmanicidal efficacy. Indeed, there is substantial overlap between miltefosine’s immunomodulating actions measured in vitro (listed above) and successful host defense in experimental visceral leishmaniasis in vivo. The latter is T cell dependent, mediated by macrophages activated by multiple cytokines, including IFN-γ, and requires induction of the macrophage’s primary microbicidal mechanisms [9]; reviewed in [11]). Therefore, in view of the clinical interest in miltefosine [4], we tested this agent in L. donovani-infected mice deficient in T cells, IFN-γ, and specific macrophage killing pathways. Our goal was to learn whether endogenous immunologic mechanisms are required for or regulate the efficacy of this new antileishmanial agent.

Materials and Methods

Animals. Euthymic and athymic (nude) BALB/c mice and normal C57BL/6 and IFN-γ gene knockout (GKO) mice (bred on a C57BL/6 background) were obtained from Charles Rivers Laboratories (Wilmington, MA). Respiratory burst-deficient gp91phox−/− (X-linked chronic granulomatous disease [X-CGD]) mice and in-
ducible NO synthase (iNOS) KO mice and their wild-type littermates, all on C57BL/6 × 129/Sv backgrounds, were originally provided by M. Dinuair and C. Nathan, respectively [9]. Both male and female GKO, X-CGD, and iNOS KO mice were used; all other mice were females. Mice were aged 8–15 weeks when challenged with L. donovani.

**Visceral infection.** Groups of 4–6 mice were injected via the tail vein with 1.5 × 10⁷ hamster spleen-derived L. donovani amastigotes (1 Sudan strain) [9]. Visceral infection was followed microscopically by use of Giemsa-stained liver imprts, and liver parasite burdens were measured by counting in a blinded fashion the number of amastigotes per 500 cell nuclei and multiplied by liver weight (in milligrams) (Leishman-Donovan units [LDU]) [9]. The histologic reaction in the liver was assessed by use of formalin-fixed stained tissue sections. Granuloma formation at infected foci was scored as none, developing, or mature [9, 11].

**Response in mice lacking macrophage microbicidal mechanisms.** Since both nude and GKO mice responded to treatment, it appeared unlikely that macrophage activation, induced by T cell-derived cytokines and expressed as enhanced microbicidal effects [11], was necessary for miltefosine’s action to proceed. Nevertheless, miltefosine primes or directly stimulates the macrophage’s 2 basic microbicidal mechanisms [6–8], the generation of respiratory burst (phagocyte oxidase [phox])–derived reactive oxygen intermediates (ROIs) and iNOS-derived reactive nitrogen intermediates (RNIs) [9, 10]. Since both ROIs and RNIs induce antileishmanial effects [9, 12], we next treated mice deficient in phox or iNOS, as well as mice deficient in both mechanisms (X-CGD mice treated with AG [9]). In comparison with control C57BL/6 and wild-type mice, the response to miltefosine was entirely retained in these 3 groups of deficient mice (table 1).

**Role of tissue granuloma in the response to miltefosine.** In livers of normal animals, infiltrating T cells and blood monocytes are assembled into granulomas surrounding L. donovani-parasitized macrophages (Kupffer cells) [11]. This histologic response is evident 2 weeks after challenge, requires T cells and monocytes, and is initially regulated by cytokines (e.g., IFN-γ) and inflammatory products, including both ROIs and RNIs [9, 11]. Thus, because the infected microenvironment was populated with both recruited cells and mediators at the time

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Table 1. Response to miltefosine (MILT) in normal and deficient mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>Liver parasite burden (LDU)</th>
<th>% killing²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day +7</td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>None</td>
<td>1408 ± 142</td>
<td>1625 ± 123</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>391 ± 38</td>
<td>72</td>
</tr>
<tr>
<td>nude BALB/c</td>
<td>None</td>
<td>2722 ± 205</td>
<td>3946 ± 323</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>999 ± 104</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>None</td>
<td>2163 ± 221</td>
<td>2766 ± 302</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>375 ± 49</td>
<td>83</td>
</tr>
<tr>
<td>GKO</td>
<td>None</td>
<td>3264 ± 333</td>
<td>5225 ± 335</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>928 ± 139</td>
<td>72</td>
</tr>
<tr>
<td>X-CGD</td>
<td>None</td>
<td>3399 ± 328</td>
<td>3291 ± 175</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>515 ± 93</td>
<td>85</td>
</tr>
<tr>
<td>x-CGD + AG</td>
<td>None</td>
<td>4326 ± 318</td>
<td>3812 ± 214</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>612 ± 107</td>
<td>86</td>
</tr>
<tr>
<td>wild-type</td>
<td>None</td>
<td>2322 ± 204</td>
<td>2145 ± 197</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>307 ± 45</td>
<td>87</td>
</tr>
<tr>
<td>iNOS KO</td>
<td>None</td>
<td>3306 ± 234</td>
<td>4092 ± 354</td>
</tr>
<tr>
<td></td>
<td>MILT</td>
<td>248 ± 39</td>
<td>92</td>
</tr>
</tbody>
</table>

NOTE. Normal C57BL/6 mice served as controls for gene knockout (GKO) and X-linked chronic granulomatous disease (X-CGD) animals; wild-type littermates were controls for inducible nitric oxide synthase (iNOS) KO mice. Results from 2–3 experiments show mean ± SEM values for LDUs for 8–16 mice/group at time treatment was started (day 0, 2 weeks after infection) and 7 days later (day +7). LDU, Leishman-Donovan units; AG, aminoguanidine treatment.

² % killing = [(day 0 LDU – day +7 LDU)/day 0 LDU] × 100. In 4 groups of deficient mice, MILT-induced % killing on day +7 was not significantly different (P > 0.05) from that of treated control mice.
Figure 1.  A–C, Histologic appearance of livers 2 weeks after infection on day 0 of miltefosine treatment. Granulomas develop at parasitized foci (arrows) in normal C57BL/6 controls (A); however, little or no cellular reaction is present at infected Kupffer cells (arrows) in either nude (B) or interferon (IFN)-γ gene knockout (GKO) mice (C). Appearance of livers from 2-week-infected X-linked chronic granulomatous disease and inducible nitric oxide synthase knockout mice was similar to (C) [9]. D and E, 1 week later (day +7) in normal C57BL/6 mice, granuloma assembly has developed further in untreated mice (D) and accelerated to mature granuloma stage (well-developed mononuclear cell mantle [11]) in miltefosine-treated animals (E). Original magnification: A, D, and E, ×315; B and C, ×500.
Miltefosine was given, the developing granuloma itself might play a facilitating role in responsiveness to therapy. However, none of the 4 deficient hosts tested in this study showed a discernible granulomatous response at the time of treatment (figure 1), indicating that miltefosine's efficacy was also granuloma independent. Nonetheless, in normal C57BL/6 mice, the already-developing granulomatous response to \textit{L. donovani} [11] was accelerated coincident with miltefosine treatment (figure 1D, 1E). Two days after treatment ended (day +7), 21% ± 4% versus 69% ± 5% of infected liver foci (\(P<.05\)) were scored as mature granulomas in untreated versus treated C57BL/6 mice (\(n = 5\) mice/group).

**Discussion**

We tested miltefosine for 3 reasons: (1) to examine whether its immunomodulatory effects in vitro [5–8] were related to its intracellular leishmanicidal action in vivo; (2) to determine whether specific, well-characterized host antileishmanial mechanisms [11] regulate in vivo therapeutic responsiveness; and (3) to extend an ongoing analysis of the effects of clinically useful antileishmanial agents (pentavalent antimony [Sb] and amphotericin B [AmB]) in immunologically intact versus deficient hosts [13–15]. In addition, we sought to gather potential experimental support for testing miltefosine in T cell– and/or IFN-\(\gamma\)-deficient patients with kala-azar, such as those with AIDS-related infection [16].

Despite the ability of miltefosine, a membrane-stimulating ether lipid analogue, to directly induce a variety of immunologic and inflammatory effects in vitro–isolated mononuclear cells and macrophages [5–8], such activating, primarily cytokine-related prohost defense effects were not required for antileishmanial action in vivo. Specifically, miltefosine was fully active in parasitized tissue devoid of T cells, endogenous IFN-\(\gamma\), primary macrophage microbicidal mechanisms, and granulomas, the latter representing the key inflammatory structure in which the preceding factors are assembled [11].

Miltefosine’s capacity to kill visceral \textit{L. donovani}, independent of both host T cells and IFN-\(\gamma\)-regulated antileishmanial mechanisms, is similar to that of AmB [13, 15] but differs from the action of Sb, the current conventional therapy for kala-azar except in parts of India. In contrast to both miltefosine and AmB, for example, Sb is entirely ineffective in T cell-deficient nude mice [13]. In related experiments, Sb also failed to induce leishmanicidal activity in GKO mice, pointing to endogenous IFN-\(\gamma\) as the T cell–dependent cofactor that regulates responsiveness to Sb [15]. In contrast, but consistent with intact efficacy in nude mice, both AmB [15] and miltefosine (this report) are fully active in the same GKO mice.

Miltefosine can induce or enhance macrophage secretion of RNIs and ROIs [6–8], molecules that act to kill intracellular \textit{L. donovani} [9, 12]. However, since treatment was effective in iNOS KO mice and in both untreated and AG-treated X-CGD mice, neither mechanism appears involved in the host response to miltefosine. iNOS KO and X-CGD mice also respond normally to Sb and AmB [15]; thus, these 2 primary microbicidal pathways are probably not required for in vivo expression of antileishmanial drug–induced efficacy. However, interaction with a macrophage antimicrobial pathway unrelated to iNOS or phox is still possible [10], and the parasitized macrophage is also not necessarily an inert participant in the response to antileishmanial treatment. For example, the efficacy of Sb is augmented in the presence of macrophages, and this effect can be enhanced still further if macrophages are first activated in vitro [14, 17].

In addition, experimental and clinical results with immunochemotherapy using Sb and exogenous cytokines (IFN-\(\gamma\) or its inducer, interleukin [IL]–12 [11, 14]) suggest that a cytokine-dependent, macrophage-activating immune response to \textit{L. donovani} can act synergistically with chemotherapy and enhance treatment efficacy. In this “two-hit” setting, the cytokine contribution would presumably involve macrophage activation and likely be iNOS- and/or phox-dependent [9, 10]; the drug contribution would presumably be independent of the latter 2 mechanisms (table 1 [15]). Although combining AmB with exogenous IFN-\(\gamma\) or IL-12 also increases antifungal activity [18], neither miltefosine nor AmB has as yet been similarly tested with cytokines in this model; thus, it is possible that the preceding exogenous cytokine-antileishmanial drug synergy may be germane only to Sb [14].

Since its activity was intact in T cell–deficient nude mice, miltefosine may also prove useful as an oral alternative for treating kala-azar in T cell–suppressed or –depleted patients, including those with AIDS-associated infection [16]. In addition, this new agent may potentially represent a convenient oral form of maintenance therapy in such patients to prevent relapse.

**Acknowledgments**

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**References**

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