Immunoenhancement Combined with Amphotericin B as Treatment for Experimental Visceral Leishmaniasis

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To determine if stimulation of Th1-cell-associated immune responses, mediated by interleukin 12 (IL-12) and gamma interferon (IFN-\(\gamma\)), enhance the antileishmanial effect of amphotericin B (AMB), *Leishmania donovani*-infected BALB/c mice were first treated with (i) exogenous IL-12 to induce IFN-\(\gamma\), (ii) agonist anti-CD40 monoclonal antibody (MAb) to maintain IL-12 and induce IFN-\(\gamma\), or (iii) anti-IL-10 receptor (IL-10R) MAb to blockade suppression of IL-12 and IFN-\(\gamma\). In animals with established visceral infection, low-dose AMB alone (two injections of 1 mg/kg of body weight; total dose, 2 mg/kg) killed 15 to 29% of liver parasites; by themselves, the immunointerventions induced 16 to 33% killing. When the interventions were combined, the leishmanicidal activities increased 3.4-fold (anti-CD40), 6.3-fold (anti-IL-10R), and 9-fold (IL-12) compared with the activities of AMB plus the control preparations; and overall killing (76 to 84%) approximated the 84 to 92% killing effect of 7.5-fold more AMB alone (three injections of 5 mg/kg; total dose, 15 mg/kg). These results suggest that strengthening the host Th1-cell response may be a strategy for the development of AMB-sparing regimens in visceral leishmaniasis.

Macrophages infected in vitro with the protozoan *Leishmania donovani* respond to treatment with either pentavalent antimony (Sb) or amphotericin B (AMB), with rapid elimination of intracellular amastigotes (3, 21). For both drugs, this in vitro leishmanicidal effect is direct, in that it is expressed in unstimulated resident macrophages and does not require the presence of host T cells or exogenously applied, macrophage-activating cytokines. Nevertheless, in mice experimentally infected with *L. donovani*, an agent of visceral leishmaniasis, the in vivo efficacies of Sb and AMB are quite different, depending upon the state of the host’s antileishmanial immune response (21, 22). This response, which culminates in macrophage activation and intracellular killing, is T cell dependent, is primarily regulated by Th1-cell-type cytokines (interleukin 12 [IL-12] and gamma interferon [IFN-\(\gamma\)], and involves mononuclear cell recruitment and granuloma assembly at infected tissue sites (27, 28).

Expression of the in vivo leishmanicidal effect of Sb in parasitized tissues is not direct, but, instead, requires the preceding mechanism with interdigitation of (i) T (Th1) cells and T-cell costimulation via CD40L and CD40, (ii) IFN-\(\gamma\) and allied activating, inflammatory cytokines (IL-12 and tumor necrosis factor), and (iii) influxing blood monocytes as primary effector cells within epithelioid granulomas (21, 25–27; H. W. Murray, C. M. Lu, E. B. Brooks, R. E. Fichtl, J. L. DeVechio, and F. P. Heinzel, submitted for publication). In clear contrast, AMB retains its leishmanicidal effect in animals deficient in T cells as well as those devoid of each of the host immune factors or mechanisms mentioned above (22, 25–27; Murray et al., submitted). Thus, while AMB’s fungicidal action may be supported by or involve induction of cytokine expression, inflammatory mediators, and cell activation (4–6, 16, 19, 33, 39–41), its experimental intracellular antileishmanial effect is independent of the host immune response and therefore is apparently direct.

Previous studies took advantage of Sb's dependence on immunologic responses, particularly those mediated by IFN-\(\gamma\) (26), by pairing Sb with treatments which raise the level of T-cell reactivity, enhance Th1-cell-type cytokine effects, and/or stimulate target cell (macrophage) activation. Such treatments included coadministration of (i) exogenous IFN-\(\gamma\) to induce macrophage activation (20), (ii) exogenous IL-12 to induce endogenous IFN-\(\gamma\) (as well as IFN-\(\gamma\)-independent effects) (25), and (iii) monoclonal antibodies (MAbs) which stimulate endogenous IL-12 and IFN-\(\gamma\) secretion via different mechanisms: anti-IL-10 receptor (IL-10R) MAb (which blocks IL-10’s suppressive effects on Th1-cell responses [29]) and agonist anti-CD40 MAb (which ligates CD40 and mimics CD40-CD40L costimulation [Murray et al., submitted]). Each of the preceding treatments synergistically increased Sb’s efficacy in mice with established *L. donovani* infections, and one of these approaches, the use of Sb plus IFN-\(\gamma\), has been tested clinically (27).

Even though AMB’s killing of *L. donovani* does not require a host immune response, we reasoned that similar targeting of the Th1-cell mechanism might increase its efficacy and permit lower doses to be used with comparable activities. Since AMB has emerged as effective, albeit arduous, treatment for human visceral leishmaniasis (kala azar) (27), it seemed worthwhile to determine if combination immunochemotherapy with AMB might have therapeutic potential for the treatment of visceral infection.

**MATERIALS AND METHODS**

Mice. Female BALB/c mice (weight, 20 to 30 g) were purchased from Charles River Laboratory (Wilmington, Mass.). Some of these mice were injected sub-
cutaneously once weekly for 4 weeks with 0.2 ml of saline containing 1.5 × 10⁷ heat-killed L. major promastigotes (HKLMP). This procedure induces a cross-reacting Th2-cell-associated response upon subsequent challenge with viable L. donovani (24), BALB/c IL-10⁻/⁻ and BALB/c IL-10 (human IL-10) transgenic mice were produced and bred at DNAX Research Institute of Molecular and Cellular Biology (Palo Alto, Calif.), as described previously (29). These mutant mice were 10 to 17 weeks old when they were challenged with L. donovani, and males and females were used in a random fashion (29).

Vesicular infection. Groups of three to five mice were infected via the tail vein with 1.5 × 10⁷ hamster spleen-derived L. donovani amastigotes (1 Sudan strain) (29). Vesicular infection was monitored microscopically by using Giemsa-stained liver imprints, in which liver parasite burdens were measured by blind counting of the number of amastigotes per 500 cell nuclei × liver weight (in milligrams) (Leishman-Donovan units [LDU]) (29).

AMB treatment. The liver parasite burdens were determined 2 weeks after infection (day +14), and a parallel group of mice then received no treatment or intraperitoneal injections of AMB (Genesia Laboratories Ltd., Irvine, Calif.). Optimal treatment consisted of 5 mg/kg of body weight, given on days +14, +16, and +18 (total dose, 15 mg/kg) (22); in the suboptimal regimen, 1 mg/kg was given on days +14 and +17 (total dose, 2 mg/kg). The mice were killed on day +21.

Combination treatments. Immunoenhancing treatments, whose antileishmanial effects have already been characterized in this model (25, 29, 30; Murray et al., submitted), were started on day +12, 2 days before AMB treatment. This 48-h period was selected to allow induction of some immunologic effect at the time that AMB treatment was begun. With the exception of anti-IL-4 (17, 24), each of the treatments tested induces a leishmanicidal effect by itself (25, 29; Murray et al., submitted). Therefore, except for anti-IL-4, previously defined suboptimal doses were used in combination with low-dose AMB (Genesia Laboratories Ltd., Irvine, Calif.). Optimal treatment consisted of 5 mg/kg of body weight, given on days +14, +16, and +18 (total dose, 15 mg/kg) (22); in the suboptimal regimen, 1 mg/kg was given on days +14 and +17 (total dose, 2 mg/kg). The mice were killed on day +21.

Effect of IL-12 coadministration. IL-12 likely initiates and maintains the antileishmanial Th1-cell-type response (8, 25, 35) and was used here to induce endogenous IFN-γ and downstream macrophage activation (23). As shown in Table 1, treatment with low-dose AMB (two injections of 1 mg/kg; total dose, 2 mg/kg) alone resulted in 19% killing of liver parasites on day +21. Use of this AMB regimen in combination with a suboptimal dose of IL-12, which by itself contributed a 33% leishmanicidal effect, increased the level of parasite killing to 82%. This leishmanicidal activity was ninefold higher than that in mice treated with saline-loaded pumps plus AMB (9% killing) and was comparable to the 92% killing in control mice given the higher-dose AMB regimen. The latter involved the administration of 7.5-fold more drug (three injections of 5 mg/kg; total dose, 15 mg/kg).

CD40 ligation. T-cell costimulation via CD40-CD40L signaling is a primary stimulus for IL-12 generation by antigen-presenting cells (38), and CD40L is required for a Th1-cell-associated response to and control over visceral L. donovani infection (Murray et al., submitted). CD40 ligation, induced by injection of agonist anti-CD40 MAb, enhances IL-12 and IFN-γ secretion (9, 13; Murray et al., submitted) and in normal mice provokes leishmanicidal activity dependent upon both cytokines (Murray et al., submitted). Therefore, low-dose AMB was next tested with anti-CD40-treated BALB/c animals (Table 2). Use of a single injection of a suboptimal dose of anti-CD40 in combination with low-dose AMB increased the level of killing to 84%, higher than that induced by either MAb (22%) or AMB (29%) alone and 3.4-fold higher than the 25% killing in mice treated with control IgG plus AMB.

Effect of AMB in a predominant Th2-cell-type cytokine environment. The downregulating Th2-cell-associated cytokines, IL-4 and IL-10, disable both the afferent and efferent limbs of the Th1 response, suppressing IL-12 and IFN-γ and deactivating macrophages (18, 27, 29, 36). In experiments conducted before determination of whether inhibition of IL-4 or IL-10 enhances drug efficacy in normal animals, AMB was tested in BALB/c mice manipulated to express a predominant Th2-cell-type response and a noncurring phenotype: (i) normal mice immunized with HKLMP, which cross-react to challenge with
mice were given optimal AMB treatment (three injections of 5 mg/kg) per time point. HKLMP-treated and IL-10 transgenic mice showed normally, with 82 to 93% parasite killing demonstrated on day 0 versus the value on day 14. The serum IL-12p40 level on day 0 was 0.2 ng/ml) relative to that on day 14 (3.6 ng/ml) was found in normal controls (two experiments; data not shown), an observation consistent with AMB’s direct antileishmanial action (27). However, these results left open the possibility that inhibition of endogenous IL-4 or IL-10, leading to enhanced Th1-cell responses (18, 24, 29, 30, 32, 36), could be combined with and increase the effect of the drug. To test this hypothesis, normal BALB/c mice were injected with anti-IL-4 or anti-IL-10R MAb on day +12 and were then treated with low-dose AMB on days +14 and +17. The use of anti-IL-4 in combination with low-dose AMB did not increase the level of parasite killing on day +21 (two experiments; data not shown), an observation consistent with prior findings that, even though IL-4 is induced (17), IL-4 exerts little or no suppressive role in the mouse model of L. donovani infection (7, 10, 11, 14, 15, 17, 27, 34). In contrast, endogenous IL-10 is a primary regulator of the outcome of L. donovani infection (29, 31). IL-10 acts in a pansen suppressive fashion to deactivate macrophages by inhibiting virtually all components of the activating Th1-cell mechanism and may directly impair the macrophage effector function as well (18, 29). Treatment of infected BALB/c mice with anti-IL-10R MAb, used to block the effects of IL-10, maintains IL-12p40 secretion in vivo, stimulates a 55-fold increase in serum IFN-γ levels, and induces IL-12- and IFN-γ-dependent parasite killing (30). As shown in Table 3, while a suboptimal dose of anti-IL-10R alone produced some leishmanicidal effect (16% killing), the use of anti-IL-10R in combination with low-dose AMB produced 76% parasite killing, a level 6.3-fold higher than the 12% killing induced by treatment with control IgG plus AMB.

Response to AMB in IL-10-deficient mice. In view of the effect of IL-10R blockade in normal mice, we completed this analysis by testing AMB in mice devoid of IL-10. These IL-10−/− BALB/c mice exert extraordinarily rapid control over L. donovani (29, 31), an effect dependent upon IL-12 and IFN-γ (31) and associated with increased levels of both IL-12p40 (7.9 ± 0.3 on day +14 versus 3.6 ± 0.4 ng/ml on day 0 [one experiment; n = 4 mice]) and IFN-γ in serum (Fig. 1). Since accelerated parasite killing is shown in IL-10−/− mice (29, 31), AMB was started earlier (on day +7) and tested in two low-dose regimens (two injections of 1 mg/kg versus a single injection of 1 mg/kg). The data in Fig. 2 show that the two-injection

L. donovani with exaggerated IL-4- and IL-10-dependent responses (24), and (ii) IL-10 transgenic mice, which show the effects of a sustained IL-10 response alone (10, 29). The latter included suppressed IFN-γ levels (Fig. 1); and no increase in the serum IL-12p40 level on day +14 (3.8 ± 0.2 ng/ml) relative to that on day 0 (3.6 ± 0.3 ng/ml) was found (1 experiment, n = 3 to 4 mice), whereas an increase from 3.1 ± 0.4 to 8.1 ± 1.1 ng/ml was found in normal controls (two experiments; n = 10 mice per time point). HKLMP-treated and IL-10 transgenic mice were given optimal AMB treatment (three injections of 5 mg/kg) starting on day +14. Both groups of animals responded normally, with 82 to 93% parasite killing demonstrated on day +21 (two experiments; n = 6 to 9 mice per group; data not shown).

Neutralization of IL-4 and blockade of IL-10R in normal mice. The preceding results indicated that suppressed Th1-type responses and the noncuring phenotype induced by excess IL-4 and/or excess IL-10 did not impair AMB’s efficacy, consistent with AMB’s direct antileishmanial action (27).

### Table 2. Effect of anti-CD40 MAb plus low-dose AMB treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Low-dose AMB</th>
<th>Liver parasite burden (LDU)</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day +14</td>
<td>Day +21</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>–</td>
<td>1,493 ± 116</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>1,060 ± 107</td>
<td>29</td>
</tr>
<tr>
<td>Anti-CD40</td>
<td>–</td>
<td>1,165 ± 99</td>
<td>22</td>
</tr>
<tr>
<td>Anti-CD40</td>
<td>+</td>
<td>236 ± 61^b</td>
<td>84</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>+</td>
<td>1,127 ± 99</td>
<td>25</td>
</tr>
<tr>
<td>Amb (optimal)</td>
<td></td>
<td>149 ± 38^a</td>
<td>90</td>
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</tbody>
</table>

^a At 12 days after infection, mice received no therapy or a single injection of anti-CD40 or rat IgG. Low-dose AMB (1 mg/kg) was given on days +14 and +17; optimal AMB (5 mg/kg) was given on days +14 and +17; optimal AMB (5 mg/kg) was given on days +14 and +16, and +18. The results are from two experiments and indicate the means ± standard errors of the means for a total of seven to eight mice per group at each time point.

^b P < 0.05 versus the value on day +14.

### Table 3. Effect of anti-IL10R MAb plus low-dose AMB treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Low-dose AMB</th>
<th>Liver parasite burden (LDU)</th>
<th>% Killing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day +14</td>
<td>Day +21</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>–</td>
<td>1,257 ± 55</td>
<td>0</td>
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<tr>
<td>None</td>
<td>+</td>
<td>1,078 ± 147</td>
<td>15</td>
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<tr>
<td>Anti-IL10R</td>
<td>–</td>
<td>1,058 ± 126</td>
<td>16</td>
</tr>
<tr>
<td>Anti-IL10R</td>
<td>+</td>
<td>302 ± 90^b</td>
<td>76</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>+</td>
<td>1,074 ± 99</td>
<td>12</td>
</tr>
<tr>
<td>Amb (optimal)</td>
<td></td>
<td>201 ± 30^b</td>
<td>84</td>
</tr>
</tbody>
</table>

^a At 12 days after infection, mice received no therapy or a single injection of anti-IL10R or rat IgG. Low-dose AMB (1 mg/kg) was given on days +14 and +17; optimal AMB (5 mg/kg) was given on days +14, +16, and +18. The results are from two experiments and indicate the means ± standard errors of means for a total of 9 to 11 mice per group at each time point.

^b P < 0.05 the value on day +14.

**FIG. 1.** Serum IFN-γ levels before (day 0) and after L. donovani infection in normal mice (open circles), IL-10 transgenic mice (open squares), and IL-10−/− BALB/c mice (filled circles). The result for each time point indicates the mean ± standard error of the mean from single experiments performed with IL-10−/− mice (n = 4 mice) and transgenic mice (n = 3 to 4) and from two experiments performed with normal mice (n = 6 to 10 mice in total).
regimen was considerably more active in IL-10−/− mice than in control mice and that measurable effects could also be achieved in IL-10-deficient animals by a single injection of a low dose of drug.

While AMB's experimental leishmanicidal activity does not require participation of the host immune response (27), the results presented here indicate that interventions which stimulate Th1-cell-type mechanisms (exogenous IL-12 and agonist anti-CD40) (25; Murray et al., submitted) or which free up Th1-cell-type mechanisms (anti-IL-10R) (30) enhance parasite killing in the livers of AMB-treated animals. Since the experiments were carried out with normal mice in the midst of developing a Th1-cell response (27), the therapeutic usefulness of these three interventions is likely predicated on the host's capacity to satisfactorily produce IL-12 and IFN-γ (9, 23, 24, 30; Murray et al., submitted). Since AMB's antifungal effect can also be successfully amplified by coadministration of IFN-γ, IL-12, tumor necrosis factor, or an IL-4 antagonist (12, 16, 37), this immunochemotherapeutic approach may have wider application.

In comparison to the killing induced by drug alone, IL-12 or agonist anti-CD40 in combination with AMB produced additive and possibly synergistic effects, while cotreatment with AMB and anti-IL-10R acted synergistically. The latter effect was particularly well illustrated in one of the three experiments whose results are summarized in Table 3, in which the levels of parasite killing on day +21 in mice treated with anti-IL-10R, AMB, or the combination were 0, 0, and 56%, respectively. It would also be of interest to determine the effects of treatment with anti-IL-10R (as well as IL-12 and anti-CD40) plus AMB in the spleen, an organ in which L. donovani replication may not be spontaneously controlled (8, 15).

The overall killing induced by combining the selected immunomodulatory regimens with low-dose AMB (total dose, 2 mg/kg) was high (76 to 84%) and in the range of that achieved by 7.5-fold more AMB used alone at a total dose of 15 mg/kg. Therefore, in an experimental infection, manipulation of the host's immune response in favor of the Th1-cell-associated mechanism may provide the opportunity to use AMB-sparring regimens with lower doses of drug, fewer injections, and/or a shorter treatment duration. Such an immunochemotherapeutic approach with these potential benefits has clinical appeal for three reasons: (i) AMB's well-recognized toxicity (e.g., anemia, azotemia, and electrolyte imbalances) is related to the cumulative dose (2); (ii) in human visceral leishmaniasis (kala azar), AMB is given for a prolonged 20- or 30-day period and is not necessarily well tolerated (27); and (iii) this approach takes advantage of available, endogenous host defense mechanisms which can separately control L. donovani when they are fully expressed in an unimpeded fashion.

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