Synergistic Effect of Glucantime and a Liposome-Encapsulated Muramyl Dipeptide Analog in Therapy of Experimental Visceral Leishmaniasis

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A regimen of immunostimulation with 6-O-stearoyl-N-acetylmuramyl-L- α -aminobutyryl-D-isoglutamine, a lipophilic analog of muramyl dipeptide, combined with antimonial drug therapy was evaluated in the treatment of visceral leishmaniasis of mice and hamsters. The combined treatment was found to be more effective in the elimination of *Leishmania donovani* amastigotes from infected tissue macrophages than was either of the two treatments applied individually. In mice, it was found that immunostimulation of animals prophylactically, therapeutically, or both enhanced the effects of the antimonial drug (Glucantime) administered more than 1 week after a challenge of BALB/c and C57BL/6 mice. The superiority of the combined treatment of the parasite infection was demonstrable in both short-term (14 days) and long-term (40 to 45 days) infections of the two inbred strains of mice. The combined therapy was also effective in preventing the lethal course of leishmaniasis in hamsters which succumb to disseminated disease in the absence of therapeutic intervention. The efficacy of this dual approach to the therapy of disseminated leishmaniasis of experimental animals holds promise for similar application in the treatment of similarly afflicted human populations.

Visceral leishmaniasis is a chronic protozoan infection in humans attendant with high mortality. The responsible agent, *Leishmania donovani*, parasitizes macrophages of the liver, spleen, and bone marrow (43). A paradoxical feature of this host-parasite relationship is the dual role of the macrophage as the obligate host cell and also as the principal effector cell for elimination of the parasite. Disseminated disease is difficult to clear with available chemotherapeutic drugs. Furthermore, currently available antileishmanial drugs possess significant inherent toxicity (3). Another complicating factor in treatment is the depressed immune functions exhibited by victims of disseminated leishmaniasis (5, 6, 23). Thus, a positive contribution of effective host defense mechanisms to assist chemotherapeutic drug therapy is often absent. In view of these complicating factors, there is a need for new strategies in eliciting a cure.

It is recognized that appropriate T lymphocyte-mediated responses are of paramount importance in the containment and eventual elimination of parasites from infected macrophages (20, 44, 45). Studies in humans (6, 23) and in experimental models (4, 5) indicate that, when T cell responses are suppressed during the course of infection, spontaneous cure and development of protective immunity does not occur. To circumvent immunosuppression, immunostimulation of the infected host presents itself as a logical possibility. This strategy has been utilized successfully in the treatment of localized cancers with *Mycobacterium tuberculosis* BCG (52) and more recently in the treatment of immune deficiency syndromes with thymopoietin fragments (16). More germane perhaps are the recent studies showing that immunostimulation of mice with glucan (9, 24), BCG (46), or *Corynebacterium parvum* (22) provides a

degree of protection against a challenge with *L. donovani*. It is likely that protection is due to the activation of the macrophage system by the immunopotentiator; in a prior study, we found that sustained activation of the macrophage is required for significant killing of *L. donovani* amastigotes (21).

Undesirable side effects elicited by complex immunopotentiating agents such as BCG and *C. parvum* preclude their general use in humans. During the past few years, several chemically defined immunomodulating compounds of low molecular weight have been developed. A prominent example is muramyl dipeptide (MDP), which appears to be the minimal structural component of the *M. tuberculosis* cell wall required for adjuvant activity (12). MDP increases both cell-mediated and humoral immune responses (13, 28, 32, 51). In this study, we evaluated the effect of a lipophilic MDP analog, alone or in combination with an antimonial drug (Glucantime), in the treatment of experimental visceral leishmaniasis. Since liposomes are effective carriers of entrapped materials and can act as selective delivery vehicles to the reticuloendothelial system (26), we used the liposome-entrapped MDP analog as an immunopotentiator in *L. donovani*-infected hamsters and in C57BL/6 and BALB/c mice. The data show that the treatment of infected mice and hamsters with a liposome-encapsulated MDP analog significantly enhances the antileishmanial effects of Glucantime.

MATERIALS AND METHODS

Animals. Female BALB/c and C57BL/6 mice weighing ca. 20 g (Jackson Laboratories, Bar Harbor, Me.) and female Syrian golden hamsters weighing ca. 90 g (Engle Laboratory Animals, Farmersburg, Ind.) were used in the experiments described.

Preparation of liposome (MLV)-encapsulated MDP. Dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol were purchased from Avanti Polar Lipids, Birmingham, Ala. L- α -Dioleoyl-[dioleoyl-1-¹⁴C]-phosphatidylcholine, 100

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mCi/mmol, was obtained from New England Nuclear Corp., Boston, Mass. The lipophilic muramyl dipeptide derivative 6-0-stearoyl-*N*-acetylmuramyl-*L*- α -aminobutyryl-*D*-isoglutamine (6-0-stearoyl-[Abu¹] MDP) was kindly supplied by Gordon Jones and Michael C. Venuti, Syntex Research, Palo Alto, Calif.

Multilamellar vesicles (MLV) were prepared with the lipophilic analog of MDP completely associated with the lipid bilayers (31). The MLV were prepared by first adding 1.8 mg of 6-0-stearoyl-[Abu¹] MDP in 1.8 ml of chloroform-methanol (2:1 [vol/vol]) to 42 mol of dioleoyl phosphatidylcholine of dioleoylphosphatidylglycerol and 18 mol (7:3 molar ratio) in 2.4 ml of chloroform. The solvents were then dried off by flushing with nitrogen, and finally the MDP analog with MLV was formed by hydrating and vortexing with 6 ml of phosphate-buffered saline (PBS; 137.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄ [pH 7.3]). The MDP analog with MLV was stored at 4°C until used to treat animals.

The complete association of the lipophilic MDP analog with MLV (34) was verified by spiking lipids with *L*- α -dioleoyl-[dioleoyl-1-¹⁴C]-phosphatidylcholine (0.1 μ Ci/ml [final concentration]), forming the MDP analog with MLV as described above, pelleting MLV at 100,000 $\times$ g, and analyzing the supernatant and pellet as described below. The radioactivity of the ¹⁴C-lipid was counted in a Beckman LS-7500 liquid scintillation counter, and the MDP analog was quantitated by the method of Reissig et al. (42) for analyzing *N*-acetyl amino sugar. The lipophilic MDP analog was completely (103 $\pm$ 8%) associated with the lipid. Endotoxin content was below detectable levels (0.05 ng/ml) in liposome preparations as determined by the *Limulus* amoebocyte assay (Mallinckrodt, Inc., St. Louis, Mo.). Hereafter, the liposome-encapsulated MDP lipophilic analog preparation is identified as MDP-MLV.

Drug. Antimony *N*-methylglucamine (Glucantime) in crystalline powder form was used as the antileishmanial drug and was provided by the Experimental Therapeutics Division, Walter Reed Army Institute of Research, Washington, D.C. The antimony content of the preparation was 33% by weight.

Amastigotes. *L. donovani* Sudan strain 1S was maintained by passage in Syrian golden hamsters. Amastigotes for passage and experimental use were collected from the spleens of hamsters infected 8 to 12 weeks previously (21). All manipulations were performed aseptically at 4°C in a biohazard hood as previously described (22). The final amastigote pellet was suspended in 5 ml of Hanks balanced salt solution. Amastigotes were counted by microscopic examination of Diff-Quick-stained (American Scientific Products, McGaw Park, Ill.) smears prepared by mixing dilutions of a parasite suspension with an equal volume of fixed chicken erythrocytes of known concentration. On the average, each infected spleen yielded 10⁹ amastigotes. The viability of parasites was determined by an acridine orange-ethidium bromide staining technique (35). Equal volumes (5 μ l) of parasite suspension and acridine orange-ethidium bromide were mixed, and several hundred amastigotes were scored by fluorescence microscopy. Preparations routinely contained 85 to 95% viable amastigotes.

Protocols for infection, immunostimulation, and drug therapy. MDP-MLV was administered intravenously in mice and hamsters at a concentration of 3.0 mg of MDP analog per kg per dose; the phospholipid content of liposomes per dose was calculated as 2 μ mol. Glucantime dissolved in PBS was injected intraperitoneally at a concentration of 37.5 mg/kg on days 8 through 11 after a challenge with *L. donovani*. Mice

were challenged intravenously with 10⁷ amastigotes, and the parasite burden in the livers was determined by microscopic evaluation of stained tissue imprints followed by the application of the Stauber formula (number of amastigotes/number of cell nuclei $\times$ liver weight [milligrams] $\times$ 2 $\times$ 10⁵ total amastigotes per liver) (50). The immunopotentiator (MDP-MLV) was administered in two doses before (days -4 and -2) or after (days +7 and +10) the challenge in two groups of mice. A third group received four doses of MDP-MLV (days -4, -2, +7, and +10). Infected mice were treated with Glucantime, MDP-MLV, or the two in combination according to the previously described regimens. Buffer liposomes (PBS-MLV), alone or in combination with Glucantime, were injected with the same protocols for MDP-MLV and the antimonial drug. A group of untreated mice served to ascertain an unmodified course of *L. donovani* infection. The parasite burden of control (untreated) mice 2 days after the challenge was used as the base-line infection level in subsequent calculations of drug or immunopotentiator efficacy or both. Experiments were terminated after 14 days, and results are expressed as the percent increase or decrease in the liver parasite burden as compared with the base-line infection level established at day 2 of infection. In a separate set of experiments, the long-term efficacy of single and combined therapy was determined at intervals between 2 days (base line) and 45 days of infection. MDP-MLV was administered on days -4 and -2 before the challenge. In this case, the data are presented as the total number of amastigotes per liver in each of the experimental groups of mice.

Hamsters were treated with the liposome-entrapped MDP analog either before (days -4 and -2) or after the challenge (days +7 and +10) with *L. donovani*. The hamsters were challenged intracardially with 5 $\times$ 10⁶ amastigotes and were treated with MDP-MLV or PBS-MLV alone or in combination with Glucantime as described above. Experiments were terminated after 15 days, and parasite burdens were ascertained as in infected mice. Since we were primarily interested in knowing how immunopotentiality enhances the efficacy of the antimonial drug, the results in this set of experiments are expressed as the percent change in parasite burden of hamsters treated with the combined regimen as compared with the animals treated with Glucantime alone.

Statistics. The statistical significance of differences in parasite burdens of the mice and hamsters was calculated by Student's *t* test.

RESULTS

Efficacy of immunostimulation and antimonial drug in *L. donovani*-infected mice. In short-term (14 days) and chronic infections (40 to 45 days), BALB/c and C57BL/6 mice were subjected to regimens of either liposome-encapsulated immunostimulant (MDP-MLV) therapy, Glucantime therapy, or a combination of the two treatment modalities. The data in Fig. 1 and 2 show the relative infection levels in the livers of BALB/c and C57BL/6 mice, respectively, after 14 days. Both strains of mice responded comparably to the combined MDP-MLV immunostimulation and antimonial drug treatment. No differences in the base-line infection levels of either strain of mice could be discerned in untreated mice versus mice treated with MDP-MLV before the challenge (days -4 and -2). This suggests that prior immunostimulation with MDP did not alter the characteristics of initial amastigote uptake from the circulation. After 14 days of infection, considerable differences in the parasite burdens of the mice subjected to the various treatments were apparent.

FIG. 1. Effect of MDP analog plus MLV immunostimulation and MDP analog plus MLV in a combined regimen with Glucantime therapy on an *L. donovani* infection in BALB/c mice. Combined data of two separate experiments are expressed as the percent increase or decrease in liver parasite burdens as compared with the base-line infection level 2 days after challenge. Five mice in each group were used in each of the experiments. Base-line infection value for the two experiments was $1.36 \pm 0.19 \times 10^7$ amastigotes per liver. Experiments were terminated 14 days after challenge. Two groups of mice received MDP analog plus MLV intravenously in two doses either on days -4 and -2 before the challenge or on days +7 and +10 after the challenge. A third group received four doses of MDP analog plus MLV on days -4 and -2 before the challenge and on days +7 and +10 after the challenge. Glucantime was administered intraperitoneally in four doses at a concentration of 37.5 mg/kg on days 8 through 11 after the challenge. The groups of mice receiving the combined regimen were dosed with MDP analog plus MLV and Glucantime as described for the individual treatments. Symbols: *, $P < 0.001$ (MDP-MLV-treated versus untreated mice); **, $P < 0.001$ (MDP-MLV plus Glucantime-treated versus Glucantime-treated mice); and ***, $P < 0.001$ (Glucantime-treated versus untreated mice).

The untreated mice of both the BALB/c and C57BL/6 strains developed comparable levels of infection (ca. a 10-fold increase above the day 2 base-line infection). Mice treated with MDP-MLV alone after the challenge (days +7 and +10) were not significantly different from untreated control mice. Immunostimulation of BALB/c mice before the challenge (days -4 and -2), however, resulted in day 14 parasite burdens which were ca. 45% less than the controls ($P < 0.001$), whereas a 25% decrease was observed with C57BL/6 mice. Treatment with the antimonial drug alone reduced infection levels ca. 60% in both mouse strains. Combined therapy with MDP-MLV and the antimonial drug resulted in a synergistic inhibitory effect (Fig. 1 and 2). Glucantime coupled with MDP-MLV suppressed the development of infection above base-line levels and were ca. 95% less than in mice treated alone with MDP-MLV on days 7 and 10. Indeed, prophylactic or therapeutic treatment with MDP-MLV combined with the therapeutic administration of Glucantime resulted in an *L. donovani* infection which was significantly less than in Glucantime-treated mice ($P < 0.001$) and 12- to 15-fold less than in the untreated controls. The data also indicate that four doses of MDP-MLV before and during infection (days -4, -2, +7, and +10) do not amplify antileishmanial effects above that achieved with two doses (Fig. 1 and 2).

The efficacy of similar treatment regimens was also determined in chronic infections of BALB/c and C57BL/6 mice

(Fig. 3a and b). Infection levels after 21 and 40 days in BALB/c mice and 30 and 45 days in C57BL/6 mice were significantly suppressed by the combined treatment ($P < 0.001$). Surprisingly, Glucantime treatment alone provided only temporary suppression of amastigote multiplication; at the termination of the experiment, parasite burdens in the untreated and Glucantime-treated mice were comparable. Additionally, the treatment of C57BL/6 mice with MDP-MLV alone reduced parasite burdens in the liver significantly ($P < 0.05$) 30 and 45 days after the challenge with *L. donovani*. It also should be noted that, in both long-term (40 to 45 days) and short-term (14 days) experiments, there was no significant difference in the parasite burdens of mice receiving PBS-MLV plus the antimonial drug and those treated with Glucantime alone (data not shown). Additionally, PBS-MLV alone did not modify the course of infection.

Efficacy of combined treatment of hamsters. Immunostimulation with MDP-MLV alone or in combination with Glucantime was also evaluated in the treatment of *L. donovani*-infected hamsters. In two experiments terminated after 15 days of infection, the superiority of the combined treatment was demonstrable. Hamsters receiving immunostimulation and Glucantime treatment in combination developed infection levels significantly lower than did animals treated with Glucantime alone ($P < 0.001$) (Fig. 4). Immunostimulation alone did not provide measurable protection. As in mice, PBS-MLV did not augment the antileishmanial effect of

FIG. 2. Effect of MDP analog plus MLV immunostimulation and MDP-MLV in a combined regimen with Glucantime therapy on an *L. donovani* infection in C57BL/6 mice. The base-line infection of two experiments was $2.16 \pm 0.14 \times 10^7$ amastigotes per liver. The details of the protocols for the single or combined treatment regimens are identical to those described in the legend to Fig. 1. Symbols: *, $P < 0.001$ (MDP-MLV plus Glucantime-treated versus Glucantime-treated mice); and **, $P < 0.001$ (Glucantime-treated versus untreated mice).

Glucantime. The combined therapy of hamsters, a species exquisitely susceptible to *L. donovani* infection, was remarkably effective. In the two separate trials, the combined treatment with liposome-encapsulated MDP analog administered in two doses either before (days -4 and -2) or after (days +7 and +10) the challenge and antimonial drug reduced parasite levels in the liver to undetectable levels in 33 and 60% of the hamsters, respectively. Patency of infection in our experiments was estimated as less than 10^6 parasites per liver.

DISCUSSION

In recent studies, we demonstrated the superiority of a combined therapeutic approach in the treatment of experimental disseminated leishmaniasis. *C. parvum* (22) or CP-46,665 (L. E. Adinolfi and P. F. Bonventre, Am. J. Trop. Med. Hyg., in press), a synthetic lipoidal amine, administered as the immunopotentiator and antimonial drug treatment, were more effective in reducing parasite burdens in infected mice than was antileishmanial drug therapy alone. In view of the notable immunomodulating properties of MDP and its analogs, we tested the efficacy of the lipophilic 6-0-stearoyl derivative of [Abu¹] MDP and the antimonial drug Glucantime in the treatment of visceral leishmaniasis of mice and hamsters.

MDP and analogs have been studied extensively in a variety of experimental situations. The synthetic peptide has been employed in the treatment of experimental tumors (15, 31) and for a variety of bacterial, fungal, and parasitic

infections (8, 17, 18, 25, 27, 36). A limitation in the use of soluble MDP as an immunopotentiator is the short half-life of the injected compound; it is estimated that MDP is cleared within 2 h after parenteral administration (39). In vitro, it has been demonstrated that MDP must be incubated with splenic lymphocytes for at least 4 h to exert a measurable stimulatory effect (56). During the past several years, phospholipid vesicles (liposomes) have attracted considerable interest for the selective delivery of drugs and immunopotentiators (14, 26) to tissue macrophages. Demonstrable advantages of the targeted delivery vehicle are the relatively slow release of entrapped MDP from tissues, the reduction in the quantity of glycopeptide required to achieve macrophage cytotoxicity (15, 30, 34, 51), and the augmentation of nonspecific resistance to infection (17). Furthermore, liposomes are nontoxic and nonimmunogenic (53). These attractive properties of liposomes prompted an evaluation of 6-0-stearoyl-[Abu¹] MDP encapsulated in MLV in the treatment of experimental disseminated leishmaniasis of mice and hamsters. A synergistic effect of immunostimulation and the antimonial drug was demonstrable in both BALB/c and C57BL/6 strains of mice. Several previous studies by others (11, 49, 56) have indicated that in vitro and in vivo responses to MDP vary with the strain of mouse tested. BALB/c mice are considered strong responders to MDP in in vitro lymphoproliferative assays as well as to the adjuvant effects of MDP in vivo. In contrast, C57BL/6 mice in similar assays are only weakly responsive. C57BL/6 mice, however, respond vigorously to reticuloendothelial system stimulation by MDP or MDP

lipophilic analog encapsulated in MLV (Ruth Waters, personal communication). The results of our study, however, do not show significant differences in the responses to the lipophilic analog MDP-MLV of BALB/c and C57BL/6 mice. Both strains of mice demonstrated a reduction in the *L. donovani* parasite burden when treated with MDP-MLV, although BALB/c mice appeared to respond to immunostimulation somewhat earlier than did the C57BL/6 mice (Fig. 1, 2, and 3A and B). On the other hand, both strains of mice responded equally well to combined immunostimulation with MDP-MLV and Glucantime therapy (Fig. 1 and 2).

The mechanisms underlying the degree of protection afforded mice by MDP-MLV treatment alone and the synergistic effect of the combined MDP-MLV and antimonial drug therapy are not known. Treatment with MDP and analogs enhances humoral immune responses to a variety of antigens (7, 28, 29) and stimulates B cell blastogenic responses (11, 49, 56) and T cell-mediated responses (13, 28). Since resistance to intracellular pathogens including *L. donovani* is dependent upon cell-mediated immunity (33, 40), it is likely that MDP-MLV therapy in our experiments resulted in the augmentation of macrophage- or T lymphocyte-mediated immune functions or both. Several studies suggest that macrophages are target cells for the biological effects of MDP (13, 47, 54). The peptide enhances carbon clearance in mice, and this augmented activity of the reticuloendothelial system is correlated with increased antimicrobial resistance (19, 55). MDP analogs also may enhance the spreading and adherence of macrophages (10), inhibit macrophage migration (57), and cause the release of interleukin 1, lymphokines, proteases, and prostaglandins (38, 48, 54). All of these phenomena are associated with activated macrophages (37). Since visceral leishmaniasis in humans (6, 23) and experimental animals (4, 5, 41) is associated with the suppression of cell-mediated immune responses, it is probable that the antileishmanial activity of 6-O-stearoyl-[Abu¹] MDP-MLV alone or in combination with Glucantime reflects an enhanced cell-mediated immunity. The synergism of the MDP analog and drug may be due to two independent but interactive antileishmanial activities. It is also possible that the MDP analog, by augmenting membrane functions of tissue macrophages, may permit the accelerated killing of amastigotes by the endocytic accumulation of the antimonial drug within parasitophorous vacuoles. Whatever the mechanism, it is evident that the combined action of 6-O-stearoyl-[Abu¹] MDP-MLV and Glucantime is synergistic (Fig. 1 and 2); MDP-MLV treatment (days +7 and +10) provided no measurable therapeutic effect, whereas Glucantime reduced infection to ca. 50% of controls. The combined treatment, in contrast, reduced parasite burdens below the day 2 base-line infection level. The data presented also suggest that the synergistic action of MDP-MLV immunostimulation and Glucantime is long-lived. The chronic infection experiments (Fig. 3A and B) reveal that the prevention of severe infection in treated mice is apparent after 40 to 45 days, although Glucantime by itself elicits only a temporary halt in amastigote multiplication.

The superiority of the combined therapeutic regimen was also seen in experiments utilizing infected hamsters (Fig. 4). The fact that immunostimulation with MDP analog plus MLV and antileishmanial drug therapy confer comparable protection to hamsters as in mice is surprising. Visceral leishmaniasis in hamsters is progressive and uniformly fatal. The infection in this species resembles human infection in several aspects. Alterations in serum globulins, anemia, and immune complex deposition in the kidneys are pathophysi-

FIG. 3. Synergistic effect of MDP analog plus MLV immunostimulation and Glucantime therapy on the course of long-term *L. donovani* infections in BALB/c and C57BL/6 mice. Data are expressed as the total number of amastigotes per liver (mean plus or minus standard error of the mean). Five mice were used in all groups of mice at each time point. Mice were treated with the MDP analog plus MLV on days -4 and -2 before challenge with *L. donovani*. Glucantime was administered as described in the legend to Fig. 1. Groups of mice receiving the combined treatment were dosed as described for the individual regimens. The remainder of protocols were the same as described in the legends to Fig. 1 and 2. Experiments with untreated (○), Glucantime-treated (●), MDP analog plus MLV-treated (▲), MDP analog plus MLV and Glucantime-treated (△) BALB/c mice (A) and C57BL/6 mice (B) are shown. *, $P < 0.05$ (MDP-MLV-treated versus untreated C57BL/6 mice); and **, $P < 0.001$ (MDP-MLV plus Glucantime-treated versus Glucantime-treated BALB/c and C57BL/6 mice).

ological consequences of the parasitic infection of humans and hamsters (1, 2, 50). Therefore, the apparent cure of several hamsters in each experiment by the combined therapy is a significant and hopeful observation.

In conclusion, the data presented show that a combined regimen of MDP analog in liposomes and Glucantime in the treatment of disseminated leishmaniasis of mice and hamsters is more effective than either treatment by itself. The results indicate further that immunostimulation with MDP-MLV facilitates leishmanicidal activity of a standard chemotherapeutic drug. The innate toxicity of antimonial agents administered in high doses (as is often required to effect a cure of visceral leishmaniasis) limits their use. Thus, a need

FIG. 4. Synergistic effect of a combined regimen of MDP analog plus MLV immunostimulation and Glucantime therapy on an *L. donovani* infection in Syrian golden hamsters. Data of two experiments are expressed as the percent increase or decrease in liver parasite burdens, with the liver parasite burden of hamsters treated with Glucantime only as the base line. The Glucantime-treated hamsters developed a day 15 infection level of $8.44 \pm 0.77 \times 10^7$ (mean plus or minus standard error of the mean) amastigotes. The data are plotted with the day 15 infection level of the group treated with Glucantime only as the zero percent base line. The histogram values represent the comparative parasite burdens at day 15 of the other groups of hamsters which received the various treatments. Five hamsters per group were in each of the experiments which were terminated 15 days after the challenge. Hamsters were treated with MDP analog plus MLV in two doses at days -4 and -2 before the challenge or on days +7 and +10 after the challenge. Glucantime and the combined therapy was administered as in mice (see the legend to Fig. 1). *, $P < 0.001$ (MDP-MLV plus Glucantime-treated versus Glucantime-treated hamsters).

for effective, relatively nontoxic treatment protocols is apparent. Our study provides experimental evidence that immunostimulation combined with an antileishmanial drug is considerably more effective in the treatment of experimental visceral leishmaniasis than is either treatment alone. Future validation of the hypothesis tested here may encourage trials of this potentially superior therapeutic approach in areas endemic for human leishmaniasis.

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