Targeting of Parasite-Specific Immunoliposome-Encapsulated Doxorubicin in the Treatment of Experimental Visceral Leishmaniasis

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A parasite-specific 51-kDa protein has been isolated from the membrane of macrophages infected with *Leishmania donovani*, the causative agent of visceral leishmaniasis. Active targeting of doxorubicin to infected macrophages was studied by incorporating it in immunoliposomes prepared by grafting F(\(ab\)\(^\prime\))\(_2\) of anti–51-kDa antibody onto the liposomal surface. In a 45-day mouse model of visceral leishmaniasis, complete elimination of spleen parasite burden was achieved by doxorubicin incorporated in immunoliposome (immuno-doxosome) at a dose of 250 mg/kg/day that was given for 4 consecutive days. A similar dose of free and liposomal drug (doxosome) had 45% and 84% parasite suppressive effects, respectively. Immunodoxosome and doxosome were generally less toxic than the free drug, as determined by several clinical parameters of cardiotoxicity and liver toxicity. These results not only indicate the potential of doxorubicin as an effective chemotherapeutic agent but also establish the use of immunoliposomes as drug carrier in the therapy of leishmaniasis.

The intracellular parasite of the genus *Leishmania* causes cutaneous, mucosal, and visceral disease in humans and poses a considerable public health problem worldwide. Visceral leishmaniasis is caused by *Leishmania donovani*, which resides and multiplies within macrophages of the reticuloendothelial system [1]. The disease is characterized by chronic fever, wasting, peripheral blood cytopenia, and massive cytosplenomegaly, any of which may lead to death without specific chemotherapy [2]. However, the drugs currently in use against leishmaniasis are highly toxic and have serious adverse effects that limit their clinical application [3, 4]. To reduce deleterious effects of drugs, one of the most recent trends in pharmaceuticals is to deliver active-drug moieties specifically to their site of action. The exclusive presence of mannose receptors on macrophages has been exploited in developing the neoglycoprotein mannosyl serum albumin as an efficient macrophage-directed drug carrier [5]. Cytotoxic drugs, such as methotrexate and doxorubicin, were found to be highly effective against visceral leishmaniasis when conjugated to neoglycoproteins [6, 7]. Although the mannose receptor–mediated approach yielded promising results, it suffered from the limitation of the drug being directed not only to the infected macrophages but also to the normal macrophages, thereby causing some toxicity. However, stem cells do not express mannose receptors; therefore, even if some macrophages were affected, stem cells would be present to replenish them. Nonetheless, a better alternative approach would be the exploitation of parasite-specific antigen that might be expressed on infected macrophages for specific targeting of drugs to these cells. Toward this goal, we reported the expression and characterization of a 51-kDa *Leishmania* species–specific protein on the infected macrophage surface, as described elsewhere [8]. A few preliminary reports are
available, which indicate the possible expression of *Leishmania* antigens on the surface of infected macrophages [9, 10]. *L. donovani*-repeating phosphorylated disaccharide lipophosphoglycan epitope was expressed on the surface of murine peritoneal macrophages infected in vitro [11]. Recent efforts have turned toward the identification of protective parasite antigens recognized by T cells in leishmaniasis [12]. The obligatory intracellular localization of *Leishmania* parasites within macrophages suggests a possibility of expression of parasite-specific antigens on infected macrophage surfaces, which may serve as a modulator of host immune response.

Recent studies in our laboratory have shown a profound antileishmanial activity for doxorubicin, a widely used anticancer drug [13]. However, its therapeutic value is strongly limited by a cumulative dose-dependent cardiotoxicity [14]. It has now been established that encapsulation of doxorubicin in liposomes can reduce the toxic effects of the drug without decreasing its efficacy [15]. The largest obstacle of liposomes targeting to any specific organ is their rapid clearance by the reticuloendothelial system. However, this natural homing of liposomes to macrophages has been made to activate the tumoricidal properties of macrophages by liposome-entrapped immunomodulators [16] and to treat diseases linked to macrophage-resident microorganisms [17] and parasites that include *Leishmania* species [18]. A potential approach for the uptake of liposomal content by macrophages is to incorporate ligands capable of interacting with macrophage surface receptors. Taking into account the presence of a 51-kDa *Leishmania*-specific protein on infected macrophage surfaces and the success obtained by delivery of doxorubicin through liposomes [19, 20], we prepared liposomes bearing anti-51-kDa antibody on their surface as an alternative means of delivering doxorubicin to infected cells for the therapy of experimental visceral leishmaniasis. The aim of the present study was to isolate and purify this parasite-specific antigen and to determine whether the F(ab\(^\prime\))\(_2\) of the antibody raised against the protein antigen could be grafted onto the liposomal surface to construct drug-laden immunoliposomes for exclusive targeting to infected macrophages.

**MATERIALS AND METHODS**

**Parasites, antigens, and macrophages.** *L. donovani* strain AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with kala azar [21]. The strain was maintained in BALB/c mice by intravenous (iv) passage every 6 weeks. *L. donovani* promastigotes for use in experiments were obtained by allowing isolated splenic amastigotes to transform in parasite growth medium for 72 h at 22°C. The growth medium consisted of medium 199 (Life Technologies) supplemented with 10% (vol/vol) fetal calf serum (FCS). Soluble leishmanial antigen (SLA) was prepared from promastigotes by freeze-thawing the cell suspension, as described elsewhere [22]. *L. donovani* promastigotes were metabolically labeled with \(^{35}\)S-methionine, as described elsewhere [23]. Antiserum against SLA was raised in rabbits, according to the method described elsewhere [8]. Macrophages were collected by peritoneal lavage from mice (BALB/c; 20–25 g) that were given intraperitoneal (ip) injection of 0.5 mL of 4% thioglycollate broth 5 days before harvest and were used according to methods described elsewhere [24]. The culture medium consisted of RPMI 1640 medium supplemented with 10 mmol/L HEPES, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, and 10% FCS. More than 90% of the cell preparation was identified as macrophages by microscopic observation, and the macrophages were routinely found to be >95% viable by use of trypan blue exclusion.

**Infection of macrophages.** Macrophages were infected with freshly transformed promastigotes at a macrophage-to-promastigote ratio of 1:10 in RPMI 1640 medium for 4 h at 37°C. Unphagocytized parasites were removed by washing with medium, and cells were resuspended in RPMI 1640 medium and 10% FCS for 72 h at 37°C. For the determination of intracellular parasite numbers, cells were fixed in methanol and were stained with Giemsa stain.

**Isolation of macrophage plasma membrane proteins.** Both normal and *L. donovani*-infected macrophages (2 \(\times\) 10\(^8\)) were incubated for 10 min at 22°C with 100 \(\mu\)g of N-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin; Pierce Chemical). After extensive washing with PBS to remove excess unbound biotin, cells were lysed in 1 mL of lysis buffer (5 mmol/L Tris-HCl [pH 7.5], 0.5% Triton X-100, 25 mmol/L KCl, and 5 mmol/L MgCl\(_2\)) in the presence of protease inhibitors (0.5 mmol/L NaCl and 0.1% Nonidet P40 (buffer II) and was passed through a Con A-Sepharose column (previously equilibrated with 50 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA, 0.5 mmol/L PMSF, and 25 U/mL aprotinin (buffer I). The column was washed with 50 mL of buffer I, and the bound material was eluted with 100 mL of a linear gradient of 0–400 mmol/L NaCl in buffer I. The eluate was dialyzed against 10 mmol/L Tris-HCl (pH 7.4) containing 0.2 mol/L NaCl and 0.1% Nonidet P40 (buffer II) and was passed through a Con A–Sepharose column (1 \(\times\) 41.5 cm) previously equilibrated with 50 mmol/L Tris-HCl (pH 7.5) supplemented with 5 mmol/L MgCl\(_2\) and 30 mmol/L \(\beta\)-octylglycoside and were dialyzed against the lysis buffer before storage at \(-70°C\).

**Purification of parasite-specific 51-kDa protein.** The macrophage membrane proteins were loaded onto a diethylaminoethyl (DEAE)–cellulose column (1 \(\times\) 10 cm) previously equilibrated with 50 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA, 0.5 mmol/L PMSF, and 25 U/mL aprotinin (buffer I). The column was washed with 50 mL of buffer I, and the bound material was eluted with 100 mL of a linear gradient of 0–400 mmol/L NaCl in buffer I. The eluate was dialyzed against 10 mmol/L Tris-HCl (pH 7.4) containing 0.2 mol/L NaCl and 0.1% Nonidet P40 (buffer II) and was passed through a Con A–Sepharose column (1 \(\times\) 41.5 cm) previously equilibrated with 50 mmol/L Tris-HCl (pH 7.5) supplemented with 5 mmol/L MgCl\(_2\) and 30 mmol/L \(\beta\)-octylglycoside and were dialyzed against the lysis buffer before storage at \(-70°C\).
Figure 1. Identification of a 51-kDa parasite-specific antigen. A, Leishmania donovani–infected macrophage membrane proteins, isolated by biotinylation and streptavidin-agarose extraction, were resolved under denaturing conditions in a 10% SDS-PAGE and were silver stained (lane 1) or transferred to nitrocellulose membrane. Transferred proteins were subjected to immunoblot analysis by use of anti–soluble leishmanial antigen (SLA) IgG as the primary probe, followed by goat anti–rabbit IgG, 5-bromo-4-chloro-indol-3-yl phosphate, and Nitro Blue Tetrazolium (lane 2). Lane 3, Uninfected macrophage membrane immunoblotted with anti–SLA IgG; lane 4, infected macrophage membrane proteins treated with preimmune serum samples.

B, Macrophages were infected with 35S-methane–metabolically labeled or unlabeled parasite, and membrane proteins were isolated, resolved in 10% SDS-PAGE, transferred to nitrocellulose paper, and autoradiographed. Lane 1, uninfected; lane 2, infected with unlabeled parasites; lane 3, infected with labeled parasites; lane 4, infected with heat-killed parasites, and lane 5, infected with formalin-treated parasites. Molecular weights are indicated to the left of each panel.

equilibrated with buffer II. The bound proteins were eluted with 10 mmol/L of Tris-HCl (pH 7.4) containing 1 mmol/L NaCl, 0.1% Nonidet P40, and 1 mol/L α-methyl-D-mannopyranoside and were dialyzed against 10 mmol/L Tris-HCl (pH 7.4) containing 150 mmol/L NaCl and 0.1% Nonidet P40. The dialysate was passed through an affinity chromatography column (1 × 10 cm) containing cyanogen bromide–activated Sepharose CL-4B coupled to the IgG fraction of anti–SLA antiserum. The resins were washed twice with 10 mmol/L Tris-HCl (pH 8.0) containing 0.1% Triton X-100 and 150 mmol/L NaCl and once with 50 mmol/L Tris-HCl (pH 7.2). The bound substances were eluted with 50 mmol/L diethylamine and 0.5% deoxycholate (pH 11.5) and were dialyzed against 0.5% deoxycholate in Tris-HCl (pH 8.0).

Electrophoresis and immunoblotting. Affinity-purified normal and infected macrophage membrane proteins from ~2 × 10⁹ cells were separated by 10% SDS-PAGE under reducing conditions and were transferred to nitrocellulose membrane (0.45 μm; Schecher and Schuell) by use of a semidyry transfer apparatus (Bio-Rad). After blotting with 20% FCS, individual strips were incubated for 2 h at 37°C with antisera against SLA at a 1:800 dilution. The strips were washed 6 times with TBS (20 mmol/L Tris-HCl [pH 7.4] and 150 mmol/L NaCl) containing 0.2% NP-40, 0.1% SDS, and 0.25% deoxycholate. Then the strips were incubated for 30 min at 37°C with secondary antibody (goat anti–rabbit IgG F(ab’)₂, conjugated to alkaline phosphatase; Sigma) diluted to 1:500 in TBS containing FCS. The protein bands were developed with Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-indol-3-yl phosphate (BCIP) in 50 mmol/L Tris-HCl (pH 9.5) containing 150 mmol/L NaCl and 5 mmol/L MgCl₂. In some experiments, the nitrocellulose membranes were incubated with anti–51-kDa antibody and with the secondary antibody, instead of with the anti–SLA antibody.

Preparation of antibodies to the 51-kDa protein. Polyclonal antibody to the 51-kDa protein was raised by ip injection of 20 µg of the protein emulsified in complete Freund’s adjuvant into a male New Zealand rabbit. Three booster doses were administered at an interval of 2 weeks by injecting the protein emulsified in incomplete Freund’s adjuvant. Ten days after the fourth injection, blood samples were collected from the rabbit ear, and the anti–51-kDa antibodies were separated, according to methods described elsewhere [25].

Preparation of doxorubicin-containing liposomes (doxosome). Multilamellar liposomes were prepared with lecithin (28 µmol), cholesterol (28 µmol), L-α-phosphatidylethanolamine (10 µmol), and doxorubicin (11.2 µmol), as described.
Figure 2. Purification profile of a 51-kDa protein by SDS-PAGE and immunoblot analysis. A, Isolated membrane proteins were passed through various chromatographic columns. Eluates from DEAE cellulose (lane 1), Con A–Sepharose (lane 2), anti–soluble leishmanial antigen (SLA) IgG–Sepharose (lane 3), and anti–51-kDa–Sepharose (lane 4) were analyzed by 10% SDS-PAGE under reducing conditions and were silver stained. B, Eluates from the various chromatographic columns, after being resolved by gel electrophoresis, were transferred to nitrocellulose membrane and were subjected to immunoblot analysis with anti–SLA IgG, as described in figure 1A. Lane 1, DEAE-cellulose; lane 2, Con A–Sepharose; lane 3, anti–SLA IgG–Sepharose; and lane 4, anti–51-kDa–Sepharose. C, Eluates from the various chromatographic columns were subjected to immunoblot analysis by use of anti–51-kDa antiserum as primary probe. Lane 1, DEAE-cellulose; lane 2, Con A–Sepharose; lane 3, anti–SLA IgG–Sepharose; and lane 4, anti–51-kDa–Sepharose. Lane 5 was treated with preimmune sera.
elsewhere [26]. Aliquots of final liposome suspension were dissolved in ethanol, and the amount of doxorubicin entrapped in liposomes was determined by measuring absorbency at 480 nm, according to the method of Mehta et al. [27].

**Covalent coupling of anti–51-kDa antibody to doxosome (immunodoxosome).** Covalent coupling of the F(ab′)2 fragment of the anti–51-kDa antibody or the anti–gp63 antibody (nonspecific immunodoxosome) to phosphatidylethanolamine of doxorubicin-containing liposome was done, according to the methods of Heath et al. [28]. Doxosomes were first oxidized for 2 h at 20°C in the absence of light with sodium periodate and were separated from excess reagent by gel filtration on a Sephadex G-50 column, using 20 mmol/L borate (pH 8.4) containing 120 mmol/L NaCl as the eluting buffer. Once oxidized, doxosomes were concentrated by use of the Amicon Centricon CF-25 cone, and the amount of lipid phosphorous was measured, according to the methods of Bartlett [29]. Five to 7 μmol of lipid phosphorous per milliliter of liposomes was mixed with a 0.2-mg F(ab′)2 fragment of anti–51-kDa antibody or anti–gp63 antibody. Two moles per liter sodium cyanoborohydride (10 μL/mL) were added to this mixture and were incubated overnight at 20°C. Doxosomes then were separated on a Sepharose 6B column (1.4 cm × 35 cm), using 10 mmol/L Tris-HCl (pH 7.4) containing 150 mmol/L NaCl, 44 mmol/L sucrose, and 5 mmol/L EDTA, as the eluant. About 5% of the total added protein was covalently attached to doxosomes by use of this method.

**Uptake of immunoliposome by macrophages as a function of both antibody concentration and time.** For the uptake study, the F(ab′)2 fragment of anti–51-kDa antibody–grafted liposomes containing 125I-bovine gamma globulin were used. Bovine gamma globulin was radio iodinated by use of the chloramine T method [30] to a specific radioactivity of 3–5 × 10⁶ cpm/μg. A total of 200 μL of each liposomal suspension (2 mg of phospholipid), in which various amounts of F(ab′)2 (0–200 nmol) were grafted, was incubated with the infected macrophages (2 × 10⁵ cells in 300 μL of RPMI 1640 medium) at 37°C. After 1 h, the suspension was centrifuged (500 g for 5 min) and washed twice, and the pellet was removed for counting of radioactivity. For time-course studies, 200 μL of liposomal suspension, both regular and modified with 150 nmol of F(ab′)2 grafted onto the liposomal surface, was incubated with the infected macrophages (2 × 10⁵ cells in 300 μL of RPMI 1640 medium) at 37°C for various time periods. Then the suspension was centrifuged, washed, and counted, as described above. Non-specific uptake was determined in the presence of a 100-fold excess of the F(ab′)2 fragment of anti–51-kDa antibody. The cell protein was assayed by use of the method of Lowry et al. [31].

**Treatment of parasite-infected macrophages with free doxorubicin, doxosome, and immunodoxosome.** Promastigotes were used to infect cultures of adherent macrophages on glass cover slips (18 mm²; 5 × 10⁵ macrophages/cover slip) in 0.5 mL of 1640 RPMI medium/10% FCS at a ratio of 10 parasites/macroocyte. Infection was allowed to proceed for 4 h at 37°C, and the unphagocytized parasites were removed by washing with medium twice. After 24 h, infected macrophages were
and 10.0 (Dex experiments.

Investigation of drug toxicity. A few parameters, such as blood pathology and specific enzyme levels related to normal heart and liver function, were chosen to determine the toxic effects of the drugs in both the free and the liposomal forms. Analyses in serum were done at day 1 after treatment with various forms of drugs at the highest concentration used (i.e., 1 mg/kg/day for 4 consecutive days). Assays were performed for albumin, creatinine, blood urea nitrogen, lactate dehydrogenase (using diagnostic kits from Sigma Chemical), serum transaminases (serum glutamate pyruvate transaminase [SGPT] and serum glutamate oxaloacetate transaminase [SGOT]) and alkaline phosphatase.

RESULTS

Identification and purification of 51-kDa protein on Leishmaniasis species–infected macrophages. Previous studies showed that a rabbit polyclonal antibody to a crude soluble antigen of L. donovani promastigotes recognized a determinant expressed on the surface membrane of mouse peritoneal macrophages and human monocyte–derived macrophages infected in vitro [8]. To identify the specific protein component, membrane proteins of parasite-infected macrophages were obtained by biotinylation and streptavidin-agarose extraction and were subjected to Western blot analysis with anti–SLA antibody. Membrane proteins were first resolved in a 10%-reducing SDS-PAGE and were transferred to a nitrocellulose membrane. After blocking the residual unbound sites, the membrane was sequentially treated with anti–SLA antibody and alkaline phosphatase–conjugated goat anti–rabbit antibody. The reaction of the NBT-BCIP complex with alkaline phosphatase revealed a major 51-kDa protein in the experimental strip (figure 1A, lane 2). Uninfected macrophage membrane proteins treated similarly did not show the 51-kDa protein, although some minor bands were observed (figure 1A, lane 3). Infected macrophage membrane proteins treated with preimmune serum failed to reveal any band, which suggests the specificity of the reaction (figure 1A, lane 4). To demonstrate the authenticity of the Leishmania origin of the expressed antigen, macrophages were infected with parasites metabolically labeled with 35S-methionine. The same 51-kDa protein was observed when surface biotinylated membrane fractions of these infected macrophages, after extraction over a streptavidin-agarose column, were electrophoresed on 10%-reducing SDS-PAGE and autoradiographed (figure 1B, lane 3). Macrophages infected with heat-killed or formalin-fixed parasites did not show any band when they were reacted with anti–SLA antiserum (figure 1B, lanes 4 and 5). Similarly, uninfected macrophages (figure 1B, lane 1) and macrophages infected with unlabeled parasites (figure 1B, lane 2) also did not show any band.

To isolate the 51-kDa component, L. donovani–infected macrophage membrane proteins were subjected to a 3-step purification procedure that involved DEAE cellulose, Con A–Sepharose, and an affinity chromatography of anti–SLA antibody–Sepharose. Analysis by use of polyacrylamide gel electrophoresis

![Figure 4. Effect of doxorubicin and immunodoxosome on growth of Leishmania donovani promastigotes. Doxorubicin-equivalent concentrations (μg/mL) for free drug: 0 (●), 0.1 (■), 0.25 (▲), 0.5 (○), 1.0 (□), and 10.0 (Δ); for immunodoxosome, (□). 1 Data are mean ± SD of 3 experiments.](https://academic.oup.com/jid/article-abstract/189/6/1024/873271)
showed that the material bound to the final step of immunoaffinity chromatography and subsequently eluted with a high pH elution buffer containing deoxycholate contained a major single band of molecular weight of 51 kDa (figure 2A, lane 5). The presence of a parasite-specific antigen of similar molecular weight was demonstrated in samples obtained from different steps of purification that were separated by electrophoresis, electroblotted onto nitrocellulose membrane, and probed with anti–SLA antibody and alkaline phosphatase–conjugated second antibody (figure 2B). In the membrane fraction, as well as in various chromatographic steps, most of the anti–SLA antibody that bound nitrocellulose was associated with a 51-kDa component. However, lower molecular-weight components visible in some of the earlier fractions indicated the possibility that other minor proteins also may bind the antibody. It could also be the result of nonspecific interactions and/or degradation of the parent protein. An antiserum generated by immunizing rabbits with the purified 51-kDa protein was found to react with a single band both in the membrane fraction and in various purification fractions with mobility identical to the antigen (figure 2C). Preimmune IgG did not provide a detectable signal with the material blotted (figure 2C, lane 5).

**Stability of immunodoxosome.** The F(ab')2 fragment of the anti–51-kDa antibody was covalently coupled onto the surface of liposomes by use of the periodate method, which did not seem to affect the structural integrity of doxosome, as viewed by electron microscopy (data not shown). The lipid components and the lipid-protein ratio were such that 5% of the total amount of added protein could be covalently attached to the liposomal surface. Use of cholesterol and cardiolipin in vesicles did not produce any appreciable change in coupling efficiency. The half-life of permeation of doxorubicin from liposomes before and after coupling remained unchanged, even after 5 days at 37°C in the presence of 20% FCS.

**Uptake of immunodoxosome.** The saturability of the parasite-specific 51-kDa antigen on the infected macrophage surface was shown by incubating the macrophages with increasing amounts of F(ab')2 grafted onto the liposomal surface at 37°C. The saturation occurred with ~100 nmol of F(ab')2 grafted onto the liposome surface (figure 3A). The phagocytosis of F(ab')2 grafted liposomes by infected macrophages was time dependent and reached equilibrium at ~30 min, whereas that by normal macrophages was significantly lesser and independent of time. The process of binding between the parasite-specific antigen and F(ab')2 grafted liposomes was found to be specific in nature. Preincubation of the macrophages with excess free F(ab')2 inhibited further binding, followed by reduced uptake of immunoliposome, which was almost similar to the uptake of regular liposomes (figure 3B; P > .05).

**Toxicity of doxorubicin.** All animals given iv injection of free doxorubicin at a dose of 20 mg/kg/day for 4 consecutive days died within 10 days. Animals given free doxorubicin at a dose of 4 mg/kg/day for 4 days survived ≤45 days. However, animals given liposomal doxorubicin at a dose of 20 mg/kg/day at the same schedule did not die, as measured up to 45 days. Loss of weight, food uptake, and other behavioral changes were not studied after drug injection. Discernible toxicity in peritoneal macrophages was observed with a doxorubicin dose of 10 μg/mL after 3 h of exposure, as evidenced by gross morphology, trypan blue exclusion, and release of lactate dehydrogenase (LDH).

**Effect of doxorubicin on L. donovani promastigotes.** The effect of doxorubicin on the growth of L. donovani promastigotes is shown in figure 4. Doxorubicin was effective against L. donovani, and promastigotes could not sustain growth at concentrations ≥250 ng/mL (P < .01, vs. drug-free control), but the effect of liposomal drug was negligible, even at 1 μg/mL (P > .5, vs. drug-free control). The drug doses used in infected mice administered with

<table>
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<th>Parameters</th>
<th>Healthy mice</th>
<th>Infected mice</th>
<th>Free drug, 1 mg/kg/day</th>
<th>Doxosome, 1 mg/kg/day</th>
<th>Immunodoxosome, 1 mg/kg/day</th>
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<tbody>
<tr>
<td>LDH, units/L</td>
<td>116.4 ± 10.8</td>
<td>149.3 ± 13.5</td>
<td>364.2 ± 38.2b</td>
<td>190.4 ± 21.3</td>
<td>158.2 ± 17.1</td>
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<td>SGPT, units/L</td>
<td>18.4 ± 2.3</td>
<td>26.3 ± 3.1</td>
<td>75.4 ± 6.8a</td>
<td>24.4 ± 2.6</td>
<td>20.5 ± 2.7</td>
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<tr>
<td>SGOT, units/L</td>
<td>35.3 ± 4.1</td>
<td>45.2 ± 4.9</td>
<td>18.1 ± 2.4b</td>
<td>47.6 ± 5.1</td>
<td>40.2 ± 4.3</td>
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<tr>
<td>Alkaline phosphatase, units/L</td>
<td>44.6 ± 3.5</td>
<td>55.3 ± 4.3</td>
<td>124.3 ± 11.2b</td>
<td>58.5 ± 4.1</td>
<td>49.3 ± 4.2</td>
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<td>Creatinine, units/L</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.2b</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
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<tr>
<td>Blood urea nitrogen, mg/dL</td>
<td>18.4 ± 2.1</td>
<td>24.5 ± 2.7</td>
<td>83.8 ± 6.9a</td>
<td>20.4 ± 1.6</td>
<td>18.1 ± 1.2</td>
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NOTE. Unit of lactate dehydrogenase (LDH), micromoles of lactate formed/min; unit of serum glutamic pyruvate transaminase (SGPT) and serum glutamic oxidase transaminase (SGOT), micromoles of pyruvate formed/min; unit of alkaline phosphatase, micromoles of p-nitrophenol formed/min. All parameters were measured in the serum of animals 1 day after drug treatment at indicated doses for 4 consecutive days. Data are mean ± SD of 3 experiments.

*P < .001, vs. healthy mice.

**P < .01, vs. healthy mice.**
Effects of various forms of doxorubicin on the growth of *Leishmania donovani* amastigotes in peritoneal macrophages. Infected macrophages were treated with various concentrations of doxorubicin for 3 h at 37°C, given as free drug (○), doxosome (□), immunodoxosome (●), and nonspecific immunodoxosome (▲). Infected controls contained 6.15 ± 0.54 amastigotes/macrophage. Data are mean ± SD of 3 experiments.

Antileishmanial activity of various drug forms in *L. donovani*–infected macrophages. Inhibition of amastigote multiplication within macrophages by immunodoxosome was compared with that by free doxorubicin, doxosome, and nonspecific immunodoxosome. Infected macrophages were treated with various drug forms for 3 h at 37°C and were washed 3 times, followed by incubation in drug-free medium for an additional 20 h. This 20-h interval was used to rid the sample of dead parasites; otherwise, it remains difficult to differentiate live from dead parasites. Controls were placed in medium alone. All samples were stained, and the number of infected macrophages was determined microscopically. Immunodoxosome was the most effective of all drug forms, with an IC₅₀ of 3.1 ng/mL, compared with 490 ng/mL for free doxorubicin (P < .0001), 9.8 ng/mL for doxosome (P < .01), and 8.0 ng/mL for nonspecific immunodoxosome (P < .01) (figure 5). Suppression of *Leishmania* species was not associated with phagocytosis of liposome alone, because liposomes without doxorubicin were not suppressive (data not shown). There was no toxic effect on macrophages in vitro by doxosome and immunodoxosome, at the highest concentration of doxorubicin (100 ng/mL) used as a liposomally incorporated drug.

**In vivo antileishmanial activity of various drug forms.**

The efficacy of various forms of doxorubicin for the treatment of visceral leishmaniasis in vivo was determined by use of a mouse model. BALB/c mice (6 weeks old; ~20 g) were infected intravenously with *L. donovani* AG83 promastigotes, as described in Materials and Methods. The infection was allowed to proceed for 45 days, during which time, spleen weight increased from 120.55 ± 13.14 mg to 935.25 ± 81.72 mg (mean ± SD; P < .001). Animals were given daily iv injections of free doxorubicin, doxosome, immunodoxosome, and nonspecific immunodoxosome for 4 consecutive days at day 15 after infection, and the infection was allowed to proceed for a total of 45 days. Various drug doses (as described above) were injected at doxorubicin-equivalent concentrations, ranging from 0.5 μg/kg/day to 500 μg/kg/day. Of the drug forms tested, immunodoxosome was most efficient in suppressing spleen parasite burden, in terms of 100% parasite suppression with subsequent reduction of spleen weight to nearly normal values, at the doxorubicin-equivalent dose of 250 μg/kg/day (figure 6). Absence of parasites in the spleen of treated animals was further confirmed by culturing spleen specimens in transformation medium for 96 h at 22°C. Free doxorubicin at the same dose caused 45% reduction (P < .0001) in spleen parasite burden, whereas doxosome and nonspecific immunodoxosome caused 84% (P < .001) and 86% (P < .001) reduction, respectively (figure 6).

**Toxic effects of various drug forms.**

In an attempt to determine the toxicity of the drug and/or the delivery system itself, the levels of 4 specific enzymes (LDH, SGOT, SGPT, and alkaline phosphatase), serum albumin and creatinine levels, and blood urea levels were determined. Some of the clinical chemistry parameters of cardiac dysfunction, such as serum LDH
Figure 6. Suppression of spleen parasite burden in a murine model of experimental visceral leishmaniasis, by doxorubicin ($\Delta$), doxosome ($\triangledown$), immunodoxosome ($\triangledown$), and nonspecific immunodoxosome ($\square$). Mice were infected with $10^7$ *Leishmania donovani* promastigotes, and drugs at indicated doses were administered intravenously for 4 consecutive days 15 days after infection. Animals were sacrificed 45 days after infection. Data are mean $\pm$ SD log_{10} Leishman Donovan units (LDUs) of 6 animals. Log_{10} LDUs of infected control animals was $2.41 \pm 0.05$.

and creatinine levels, and liver dysfunction, such as serum albumin, transaminases (SGOT and SGPT), alkaline phosphatase, and blood urea nitrogen levels, were found to be significantly altered in animals treated with free doxorubicin at a dose of 1 mg/kg/day for 4 consecutive days, which indicates some type of cardiotoxicity and liver toxicity (table 1). However, doxorubicin given in the form of doxosome and immunodoxosome did not result in any cardiotoxicity or hepatotoxicity at a similar dose, as reflected by the unaltered levels of clinical parameters, compared with those for control values.

**Statistical analysis.** Results are expressed as mean $\pm$ SD of 3 separate experiments. We used analysis of variance to determine the statistical significance of intergroup comparisons. $P < .05$ was considered to be statistically significant.

**DISCUSSION**

The present study has described the potential of a drug-targeting system for specific delivery of active drug moieties to infected macrophages in the therapy of experimental visceral leishmaniasis. A widely used anticancer drug, doxorubicin, was incorporated in liposomes that were grafted with antibodies against parasite-specific antigen expressed exclusively on an infected macrophage surface (immunodoxosome). Earlier studies from our laboratory showed a profound antileishmanial activity for doxorubicin [13]. The superior efficacy of the encapsulated drug in eliminating intracellular amastigotes of *L. donovani* in both an in vitro macrophage model and an in vivo mouse model of visceral leishmaniasis demonstrates the effectiveness of this approach. Although the property of natural homing of liposomes by macrophages has rendered the liposomal doxorubicin (doxosome) much more effective than free doxorubicin, results clearly indicate a substantial increase in the efficacy of the drug-incorporated immunodoxosome, compared with doxosome. Internalization of the antibody-grafted liposomal drug through the parasite-specific 51-kDa antigen on macrophage surface has been suggested, because the uptake in vitro of the immunoliposome at 37°C is rapid and highly specific. The uptake attained equilibrium in $\sim$30 min. This in vitro specificity of antigen-antibody interaction also was noticed when it was tested in vivo. The immunodoxosome was found to be rapidly cleared from the blood circulation (half-life, $\sim1.1$ min) and taken up by the cells of the reticuloendothelial system (data not shown).

In the present study, immunodoxosome was consistently more effective than doxosome, nonspecific immunodoxosome, and free doxorubicin in causing parasite suppression in infected macrophages. Moreover, doxorubicin, given in the form of immunodoxosome, completely eliminated the spleen parasite burden in infected animals at a dose of 250 mg/kg/day for 4 consecutive days. Like many other strong anticancer drugs, doxorubicin has toxic effects, mainly hematologic and cardiac...
[38]. However, liposome entrapment has significantly reduced the cardiotoxicity, as assessed by serum LDH and creatinine levels, which are considered to be indicators of myocardial damage. In extrapolating the present dose to an 80-kg human with a surface area of 2 m$^2$ (Costeff’s rule), a total dose of 16 mg (500 μg × 80 × 4) or 8 mg/m$^2$ of surface area would be required. This dosage is far below the toxic dose of 250 mg/m$^2$ that causes myocardial toxicity, as demonstrated by subendocardial biopsies [39]. Because doxorubicin is metabolized and excreted primarily by the liver, clinical parameters of liver toxicity were assessed, which showed significantly reduced toxicity for encapsulated drugs. The mechanism by which immuno- doxosome improved the therapeutic index of doxorubicin in the treatment of leishmaniasis is presumed to be a facilitated delivery of the drug to infected macrophages of liver and spleen through parasite-specific antigen. However, prolonged presence of doxorubicin in the liver and spleen that results from entrapment of drug within liposomes also may account for the improved therapeutic activity. The combined effect of antibody against parasite-specific antigen and liposomes resulted in an increased availability of the drug for intracellular parasites. In a similar approach, attachment of anti–candidial antibody to the liposomal membrane containing amphotericin B was observed to enhance the therapeutic effect of the drug [40].

In summary, we have demonstrated specific binding and effective intracellular drug delivery to Leishmania species–infected macrophages by use of antileishmanial antibody. Because of the specificity of F(ab)$^\prime$, of antileishmanial antibody for infected macrophages and the marked effect of liposomal doxorubicin on intracellular parasites of Leishmania species, this type of antibody-mediated drug targeting may provide a method for reaching the appropriate diseased site, with reduced toxicity and enhanced specificity.

References

37. Walter K, Schult C. Alkaline phosphatase in serum, continuous essay.