

The sterol-binding antibiotic nystatin inhibits entry of non-opsonized *Leishmania donovani* into macrophages[☆]

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Abstract

Leishmania donovani is an obligate intracellular parasite that infects macrophages of the vertebrate host resulting in visceral leishmaniasis in humans, a major public health problem worldwide. The molecular mechanisms involved in internalization of *Leishmania* are still poorly characterized. We report here that cholesterol sequestration by the sterol-binding antifungal polyene antibiotic nystatin markedly inhibits binding and entry of non-opsonized *L. donovani* promastigotes into macrophages. Interestingly, these effects are not observed when serum-opsonized *L. donovani* are used for infectivity studies thus pointing the essential role of cholesterol in mediating entry of the parasite via the non-opsonic pathway. Based on our earlier results where leishmanial infectivity was shown to be sensitive to physical depletion of cholesterol from macrophages, these results indicate that the mere sequestration of cholesterol in the host plasma membrane is sufficient to inhibit the binding and entry of non-opsonized *L. donovani*. These results represent the first report on the effect of a cholesterol-sequestering agent on the entry of *Leishmania* parasites to host macrophages. More importantly, these findings offer the possibility of reevaluating the mechanism behind the effectiveness of current therapeutic strategies to treat leishmaniasis.

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Human visceral leishmaniasis is an opportunistic disease caused by the protozoan parasite *Leishmania donovani* [1]. The estimated annual number of new cases of visceral leishmaniasis is about 500,000 [1]. The current increase in leishmaniasis throughout the world to epidemic proportion coupled with increasing incidence of the disease in developed countries, and emergence of visceral leishmaniasis as an important opportunistic infection among people with human immunodeficiency-1 (HIV-1) infection [2] have created an urgency to provide treatment for this intracellular infection. *Leishmania* are digenetic protozoan parasites and exist either as extracellular promastigotes that reside

in the mid-gut of sandflies or as intracellular amastigotes adapted for survival within phagolysosomes of mammalian macrophages. Entry of promastigotes into macrophages involves recognition of specific ligands on the parasite cell surface by receptors on the macrophage cell surface and the subsequent internalization of the parasite by the macrophage. Studies on the molecular mechanisms of parasite entry have led to the identification of several candidate receptors facilitating multiple routes of entry thereby highlighting the redundancy in the entry process [3]. These include membrane proteins present on the macrophage cell surface such as the mannose–fucose receptor, receptor for advanced glycosylation end products, the fibronectin receptor, the Fc receptor (FcR), and the complement receptors such as the CR1 and CR3. The large number of different receptors responsible for the entry of the parasite into host macrophages makes it difficult to have a unique therapeutic target for the treatment of leishmaniasis.

[☆] Abbreviations: M β CD, methyl- β -cyclodextrin; FITC, fluorescein isothiocyanate.

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The entry of *Leishmania donovani* in particular and other intracellular parasites in general involves interaction with the plasma membrane of host cells. Cholesterol is a major constituent of the eukaryotic plasma membrane. The essential role of cholesterol in maintaining membrane protein function is well established [4]. This is proposed to occur either due to a specific local molecular interaction with membrane proteins, or due to alterations in the membrane physical properties induced by the presence of cholesterol [4–7], or due to a combination of both factors. We have recently shown the requirement of host membrane cholesterol in the binding and internalization of *Leishmania* promastigotes into macrophage cells [8]. This was achieved by the use of methyl- β -cyclodextrin (M β CD) which physically depletes cholesterol from membranes [9,10]. Treatment of macrophages in culture with M β CD resulted in the specific removal of membrane cholesterol and a concomitant reduction in binding and subsequent infection by *Leishmania* promastigotes [8]. If cholesterol is necessary for leishmanial infection, modulating cholesterol availability by other means could affect leishmanial infection. In this report, we have tested this proposal by treating macrophages with the sterol-binding antifungal polyene antibiotic nystatin [11–13]. Nystatin specifically interacts with cholesterol to sequester it in the membrane thereby effectively reducing the ability of cholesterol to interact with and exert its effects on other membrane components such as receptors. Our results show that sequestration of cholesterol in the macrophage membrane without its physical depletion is sufficient to inhibit leishmanial infection. Although nystatin has earlier been used on isolated leishmania parasites [14,15], this is the first report describing the effect of nystatin treatment of host macrophages on leishmanial infection.

Materials and methods

Materials. RPMI-1640 medium, M-199 medium, penicillin, streptomycin, nystatin, M β CD, and FITC were obtained from Sigma Chemical (St. Louis, MO, USA). Fetal calf serum was obtained from Gibco/BRL Life Technologies (Scotland, UK). Radiolabeled [3 H]thymidine was obtained from Dupont New England Nuclear (Boston, MA, USA).

Cells and cell culture. The murine macrophage J774A.1 (American Type Culture Collection) cells were maintained at 37 °C in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% heat-inactivated fetal calf serum [8].

Parasite culture. *Leishmania donovani* strain AG83 (MHOM/IN/1983/AG83) parasites were maintained as promastigotes at 22 °C in modified M-199 medium supplemented with 100 U/ml and 100 μ g/ml streptomycin and 10% heat inactivated fetal calf serum as described previously [16].

Cholesterol sequestration of macrophages. Cholesterol sequestration was carried out by incubating J774A.1 cells with nystatin in serum-free RPMI-1640 medium at 37 °C for 30 min followed by a wash with PBS before being exposed to parasites. Macrophages were plated at a density of 1×10^5 in 35 mm culture dishes and grown for 48 h for radiolabeled or FITC-labeled parasite-binding experiments. For Giemsa staining experiments, macrophages were plated at a density of 5×10^5 in 60 mm dishes and grown for 24 h.

Radiolabeling *L. donovani* promastigotes with tritium or FITC for binding studies. Parasites were metabolically radiolabeled with tritium as described previously [17] with some modifications. Radiolabel incorpo-

ration was carried out at a density of 1×10^7 parasites/2 ml M-199 medium in the presence of 20 μ Ci/ml [3 H]thymidine (sp. activity = 89.4 Ci/mmol) at 22 °C for 3 h. FITC-labeling of parasites was carried out as described previously [18], except that labeling was carried out 37 °C in phosphate-buffered saline (PBS).

Oposonization of *L. donovani* promastigotes. Oposonization was carried out as described earlier [19], with few modifications. Promastigotes were first radiolabeled with 20 μ Ci/ml [3 H]thymidine as described above. Radiolabeled promastigotes were suspended in mouse serum at a density of 1×10^8 cells/ml at 37 °C for 25 min in PBS. Oposonized parasites were washed once with PBS and used directly.

Infectivity assays. Promastigotes were added onto macrophage monolayers at a parasite to macrophage ratio of 10:1 in RPMI-1640 medium at 37 °C for the indicated time periods. At the end of incubation, monolayers were washed twice with PBS to remove free parasites, solubilized with 1% Triton X-100, and assayed for radioactivity using a Packard Tri-Carb liquid scintillation counter. In flow cytometric experiments, FITC-labeled parasites were used to infect macrophages for the indicated time periods. After infection, macrophages were gently scraped into PBS with 0.1% formaldehyde at 4 °C. The fluorescence from FITC-labeled parasites associated with 5000–10,000 macrophages was analyzed with a Beckman Coulter ELITE ESP flow cytometer using EXPO 32 software for data analysis. To assess the percentage of macrophages harboring the intracellular amastigote form of the parasite, macrophages were exposed to the parasites for a period of 3 h after which cell monolayers were stained with Giemsa. The number of amastigotes in macrophages was visually scored using a Leica microscope with a 100 \times oil-immersion objective.

Results

We monitored the effect of treating host J774A.1 macrophages with nystatin on the binding of *Leishmania* promastigotes. As mentioned earlier, nystatin specifically interacts with cholesterol [11–13] to sequester it in the membrane thereby effectively reducing the ability of cholesterol to interact with other membrane constituents such as receptors. In these experiments, macrophages were incubated with increasing concentrations of nystatin, followed by a wash to remove any unbound nystatin before being exposed to *Leishmania* parasites. In order to analyze the effect of nystatin treatment on the ability of *Leishmania* to infect macrophages, radioligand-binding assays with [3 H]thymidine-labeled non-opsonized parasites were carried out. As shown in Fig. 1A, treatment of macrophages with increasing concentrations of nystatin progressively reduces the binding of parasites to macrophages. Thus, a concentration of 25 μ g/ml nystatin gives rise to ~50% reduction in macrophage-parasite interaction. The effect of nystatin treatment was further analyzed by monitoring the kinetics of macrophage–parasite interaction. As shown in Fig. 1B, macrophages pretreated with 25 μ g/ml nystatin, when used as hosts against the parasite, display a time-dependent reduction in macrophage–parasite interaction with ~60% reduction when infection was monitored for 180 min.

The entry of *Leishmania* into macrophages has been attributed to multiple receptor-mediated mechanisms involving several macrophage cell surface receptors. These include receptors such as the CR1 and CR3, the mannose–fucose receptor, the fibronectin receptor, the receptor for advanced glycosylation end products, the Fc receptor,

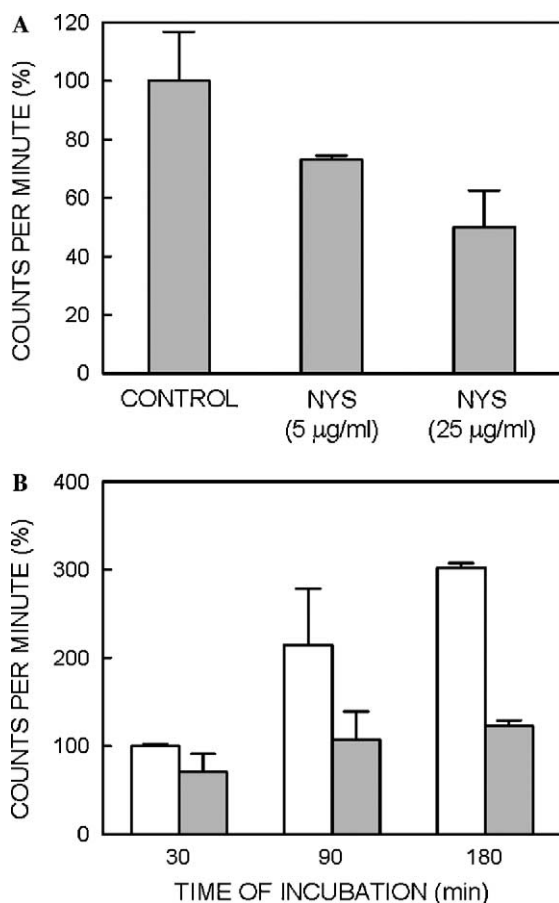


Fig. 1. Effect of cholesterol sequestration by nystatin on the extent and kinetics of binding of *Leishmania* promastigotes to host macrophages. (A) The extent of binding of radiolabeled non-opsonized promastigotes to macrophages with increasing concentrations of nystatin (NYS) is shown. Macrophages were exposed to radiolabeled parasites for 90 min at a parasite to macrophage ratio of 10:1. All values are normalized with respect to the mean counts per minute obtained for control cells. The data shown are means \pm SD of two independent experiments performed in duplicate. (B) The kinetics of binding of radiolabeled non-opsonized *Leishmania* promastigotes to control (white bars) and nystatin-treated (gray bars) macrophages is shown. Macrophages were pretreated with nystatin at a concentration of 25 μ g/ml. All values are normalized with respect to the mean counts per minute obtained for control cells after 30 min exposure to the parasite. The data shown are means \pm SD of two independent experiments performed in duplicate. See Materials and methods for other details.

and the C-reactive protein receptor. Serum-opsonized parasites are considered to predominantly bind to the CR1 and CR3 receptors on the macrophage cell surface and accomplish infection [3]. To further understand the reduced infectivity of *Leishmania* promastigotes in cholesterol-sequestered macrophages, we carried out binding studies with radiolabeled promastigotes which were opsonized with mouse serum. Fig. 2 shows that while binding of non-opsonized *Leishmania* to macrophages is reduced by \sim 50% upon treatment with 25 μ g/ml nystatin (similar to that seen in Fig. 1A), binding of opsonized *Leishmania* under similar conditions does not show any significant reduction. This result shows that cholesterol sequestration

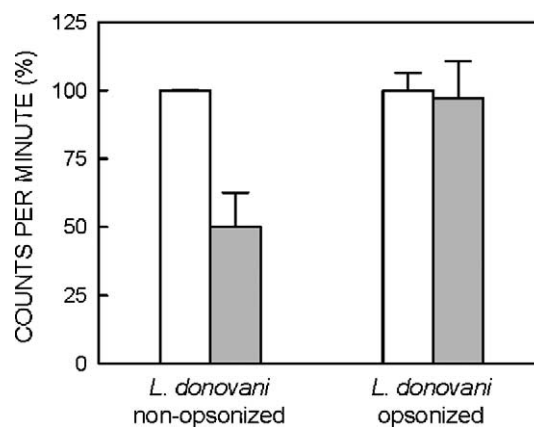


Fig. 2. Comparison of the effect of cholesterol sequestration by nystatin on the binding of non-opsonized and opsonized *Leishmania* promastigotes to host macrophages. Control (white bars) or nystatin-treated (gray bars) macrophages were exposed to radiolabeled parasites for 90 min at a parasite to macrophage ratio of 10:1. Macrophages were pretreated with nystatin at a concentration of 25 μ g/ml. All values are normalized with respect to the mean counts per minute obtained for the respective controls in the experiment. The data shown are means \pm SD of two independent experiments performed in duplicate. See Materials and methods for other details.

does not affect the route of entry undertaken by opsonized parasites. This is similar to our previous result in which we showed that the entry of opsonized *Leishmania* parasites into macrophages is insensitive to cholesterol depletion by M β CD [8]. Taken together, these results point out the commonality in the underlying mechanism associated with the inhibition in entry of non-opsonized parasites into macrophages which is a result of non-availability of membrane cholesterol. Importantly, the actual means by which membrane cholesterol is made unavailable (physical depletion using M β CD or sequestration by nystatin) therefore does not appear to be crucial for the observed reduction in infectivity.

We confirmed our results obtained with radioligand-binding experiments (Figs. 1 and 2) with flow cytometric analysis of binding of FITC-labeled promastigotes to macrophages. Fluorescent derivatization of promastigotes with FITC has previously been used as a convenient tool to accurately monitor host–parasite interaction and provides an ideal means for studying cell surface interaction phenomena since each cell is analyzed individually for its ability to bind to a fluorescent ligand which in this case is the FITC-labeled promastigote [18]. Data shown in Fig. 3A reveal a time-dependent reduction in fluorescence associated with nystatin-treated macrophages as compared to control macrophages when infection is allowed to progress for a period of 90 min. These data support our earlier conclusion (from Fig. 1) of a reduction in the ability of non-opsonized *Leishmania* promastigotes to interact with the host upon sequestration of host membrane cholesterol. However, similar analysis carried out with opsonized promastigotes (see Fig. 3B) shows no significant difference in binding of the parasite to nystatin-treated macrophages

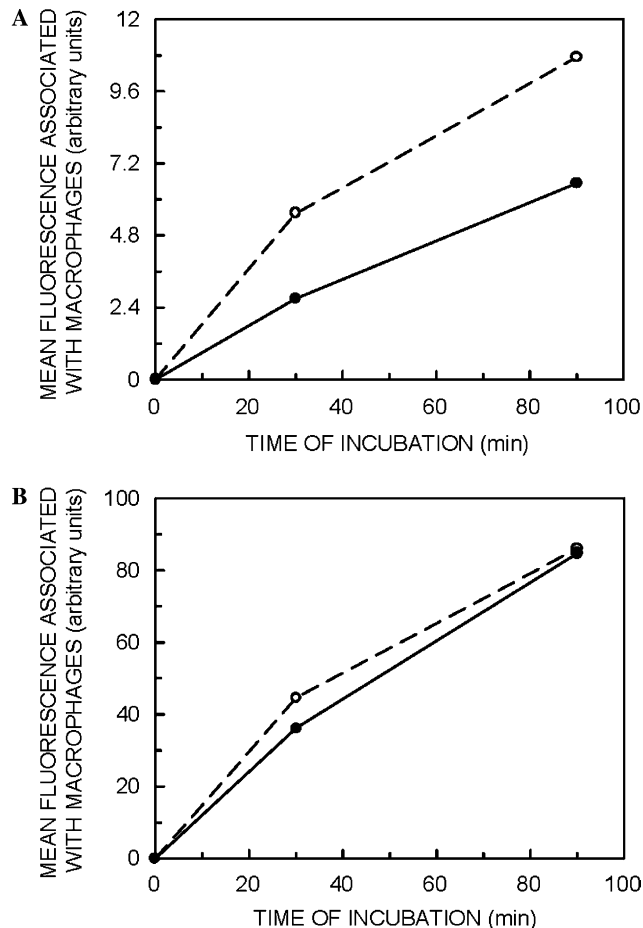


Fig. 3. Flow cytometric analysis of the effect of cholesterol sequestration by nystatin on the kinetics of binding of FITC-labeled non-opsonized and opsonized *Leishmania* promastigotes to host macrophages. Control (open circles) or nystatin-treated (closed circles) macrophages were exposed to non-opsonized (A) or opsonized (B) FITC-labeled promastigotes at a parasite to macrophage ratio of 10:1. Macrophages were pretreated with nystatin at a concentration of 25 $\mu\text{g}/\text{ml}$. The data are representative of two independent experiments performed in duplicate. See Materials and methods for other details.

monitored for the same duration of time. This is in agreement with the results obtained with radiolabeled parasites (shown in Fig. 2).

The fact that binding of opsonized *Leishmania* to nystatin-treated macrophages remains unaffected serves as an important control to suggest that the effect of cholesterol sequestration on the binding of non-opsonized parasite is not due to a general loss of viability of macrophages after nystatin treatment. This further helps stringently define the specificity of interaction of the non-opsonized form of the parasite with the host cell membrane. In a control experiment, we monitored the effect of nystatin treatment of macrophages on the uptake of another microbe, namely *Escherichia coli* DH5 α , under similar conditions as those used for *Leishmania* promastigotes. In sharp contrast to what is observed with *Leishmania*, flow cytometric analysis of the binding of FITC-labeled *E. coli* to control and nystatin-treated J774A.1 cells did not show any significant

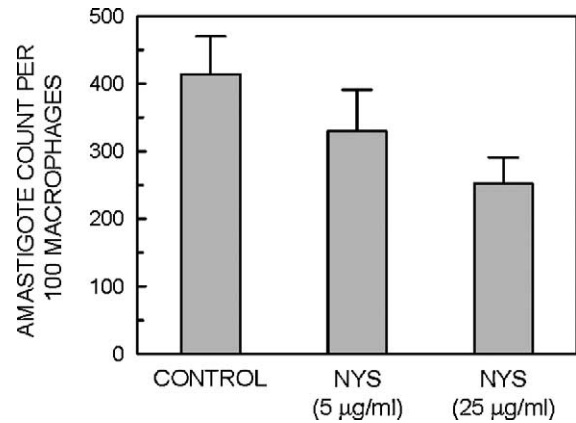


Fig. 4. Effect of cholesterol sequestration by nystatin on the intracellular amastigote load of *Leishmania* parasite in host macrophages. The count of the intracellular amastigote form of the parasite is shown in macrophages either untreated or treated with increasing concentrations of nystatin (NYS). Macrophages pretreated with nystatin were exposed to non-opsonized promastigotes at a parasite to macrophage ratio of 10:1 for 3 h. The data shown are means \pm SE of three independent experiments performed in duplicate. See Materials and methods for other details.

difference (data not shown). This points to the specificity of cholesterol-dependent interaction between *Leishmania* promastigotes and the host cell membrane.

The above results demonstrate that cholesterol sequestration leads to a reduction in the ability of non-opsonized promastigotes to bind to host macrophages. For efficient infection, binding of the parasite should be followed by internalization. During the course of infection, the reduced binding of the promastigotes should manifest in a reduction in the intracellular load of amastigotes, the intracellular form of the parasite present in macrophages. The number of amastigotes after 3 h of exposure of the parasite to nystatin-treated macrophages was determined visually after staining the infected macrophages with Giemsa. As shown in Fig. 4, treatment of macrophages with nystatin results in a concentration-dependent reduction in the number of intracellular amastigotes present (compared to control cells) with a \sim 40% reduction in the amastigotes load when macrophages pretreated with 25 $\mu\text{g}/\text{ml}$ nystatin were used as host.

Discussion

This report represents one of the first studies on the effect of agents that perturb membrane cholesterol on the binding of *Leishmania* parasites to host macrophages. As mentioned earlier, although nystatin has previously been reported to possess leishmanicidal property [14,15], this is the first report that describes the effect of treatment of host macrophages with nystatin on leishmanial infectivity. We have recently shown that the physical depletion of cholesterol from macrophages leads to inhibition in the binding and internalization of *Leishmania* promastigotes into macrophage cells [8]. Our present results with the use of the sterol-binding agent nystatin, which does not deplete

cholesterol from membranes, suggest that mere sequestration of host plasma membrane cholesterol is sufficient to inhibit leishmanial infection. Sequestration of membrane cholesterol with nystatin could lead to a reduction in the availability of free cholesterol in the host plasma membrane essential for parasite entry (see later). Taken together, these results reinforce the crucial requirement of membrane cholesterol in host cells for leishmanial infection.

The molecular mechanism of how cholesterol supports binding of the parasite and its entry into host macrophages remains a key issue and requires further investigation. Interestingly, the effects of cholesterol sequestration are abrogated when serum-opsonized parasites are used in similar experiments as carried out for non-opsonized parasites. This points toward the essential role of cholesterol in supporting entry of the parasite via the non-opsonic pathway into macrophages. The involvement of multiple membrane-bound receptors in the entry of the parasite into host cells has been mentioned earlier [3]. The modulatory role of cholesterol, an essential component in the plasma membrane of eukaryotic cells, on the function of membrane receptors, such as the oxytocin receptor [4] and the serotonin_{1A} receptor [10,20], has been previously demonstrated. These results show that perturbing the cholesterol content and/or availability in the membrane may lead to perturbation of receptor–cholesterol interactions leading to loss of receptor function.

Cholesterol is believed to modulate organization of lipids and proteins in the cell membrane [21]. It is often found distributed non-randomly in domains in membranes [22]. Recent observations suggest that cholesterol exerts many of its actions by maintaining putative membrane domains termed “lipid rafts” in a functional state [23]. Lipid rafts are thought of as lateral organizations on the plane of the membrane that are enriched in cholesterol and sphingolipids and specific proteins that act as receptors for the entry of microorganisms. As a result, pathogen entry into cells is thought to be dependent on the integrity of such regions of the membrane [24]. Importantly, cholesterol complexing agents such as nystatin have previously been shown to affect the integrity of such domains and the function of membrane proteins localized in them [25,26]. It is possible that the reduction in *Leishmania* infection upon nystatin treatment is due to alterations in the function and organization of one or more of the many receptors on the host cell surface that participate in parasite attachment and internalization [3].

On a broader perspective, our results offer the possibility of reevaluating the mechanism behind the effectiveness of current therapeutic strategies to treat leishmaniasis. Among the popular clinically prescribed therapeutic drugs to treat visceral leishmaniasis is amphotericin B [27,28] that like nystatin is another sterol-binding antibiotic [11,13]. Although the development of amphotericin B as a therapy against leishmaniasis has its origin in the discovery that it is a potent leishmanicidal agent [29,30], it is possible that its

effectiveness in vivo is partly based on its ability, like nystatin, to sequester cholesterol in the host membrane thereby reducing macrophage–parasite interaction.

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