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Cholesterol is required for *Leishmania donovani* infection: implications in leishmaniasis

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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, which is usually fatal if untreated. The molecular mechanisms involved in host–parasite interaction leading to attachment on the cell surface and subsequent internalization of the parasite are poorly characterized. Cholesterol is a major constituent of eukaryotic membranes and plays a crucial role in cellular membrane organization, dynamics, function, and sorting. It is often found distributed non-randomly in domains in membranes. Recent observations suggest that cholesterol exerts many of its actions by maintaining a specialized type of membrane domain, termed "lipid rafts", in a functional state. Lipid rafts are enriched in cholesterol and sphingolipids, and have been thought to act as platforms through which signal transduction events are coordinated and pathogens gain entry to infect host cells. We report here that cholesterol depletion from macrophage plasma membranes using methyl-β-cyclodextrin (MβCD) results in a significant reduction in the extent of leishmanial infection. Furthermore, the reduction in the ability of the parasite to infect host macrophages can be reversed upon replenishment of cell membrane cholesterol. Interestingly, these effects were not observed when parasites were serum-opsonized, indicating a specific requirement of cholesterol to mediate entry via the non-opsonic pathway. Importantly, we show that entry of *Escherichia coli* remains unaffected by cholesterol depletion. Our results therefore point to the specific requirement of plasma membrane cholesterol in efficient attachment and internalization of the parasite to macrophage cells leading to a productive infection. More importantly, these results are significant in developing novel therapeutic strategies to tackle leishmaniasis.
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Keywords: *Leishmania*; Serum-opsonized *Leishmania*; Membrane cholesterol; Methyl-β-cyclodextrin; Cholesterol depletion; Therapeutics

1. Introduction

Leishmania are protozoan parasites that are responsible for substantial public health problems, especially in tropical and subtropical regions. The parasite is responsible for the disease leishmaniasis, which is usually fatal if left untreated [1,2]. In a recent survey, 88 countries have been declared as leishmaniasis-endemic [1]. The estimated annual number of new cases of leishmaniasis is thought to be 2 million [3] and 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 [3] have created an urgency to provide treatment for this intracellular infection. Studies on the molecular mechanisms of parasite entry have led to the identification of several candidate receptors facilitating multiple routes of entry and thus highlighting the redundancy in the entry process. These include macrophage cell surface receptors, such as the CR1 and CR3, the mannose–fucose receptor, the fibronectin receptor, the receptor for advanced glycosylation end products, the Fc receptor, and the C-reactive protein receptor [2]. However, the large number of different receptors responsible for the attachment and internalization of the parasite to macrophages have contributed to the fact that no single panacea has yet been developed for the treatment of leishmaniasis.

Abbreviations: BCA, bicinchoninic acid; MβCD, methyl-β-cyclodextrin

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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. Several lines of investigations point to the essential role of cholesterol in maintaining membrane protein function through specific lipid–protein interactions [4]. In addition, cholesterol is thought to modulate organization of lipids and proteins on the cell surface [5]. It is often found distributed non-randomly in domains in membranes [6,7]. Recent observations suggest that cholesterol exerts many of its actions by maintaining a specialized type of membrane domain, termed "lipid rafts", in a functional state [8]. 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 are implicated in cell signaling and receptor-mediated entry of microorganisms. The integrity of such regions of the membrane is thought to be crucial to regulate signal transduction events [9] and entry of pathogens into the cell [10]. These observations prompted us to investigate the role of plasma membrane cholesterol in the infection of *L. donovani* in macrophages.

2. Materials and methods

2.1. Cells and cell culture

Murine macrophage cell line J774A.1 (American Type Culture Collection) was maintained at 37 °C in RPMI-1640 medium (Sigma) containing 10% heat-inactivated fetal calf serum (Biological Industries, Israel) as described previously [11].

2.2. Parasite culture

Leishmania donovani strain AG83 (MHOM/IN/1983/AG83) promastigotes were maintained at 24 °C in modified M-199 medium (Gibco/BRL) supplemented with 100 units/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 10% heat-inactivated fetal calf serum.

2.3. Modulation and estimation of cholesterol content

Cholesterol depletion was carried out by incubating J774A.1 cells grown on culture dishes with 5 or 10 mM methyl-β-cyclodextrin (MβCD; Sigma) in serum-free medium at 37 °C for 30 min [12,13]. The macrophage cells were plated at a density of 1×10^5 in 35 mm culture dishes and grown for 48 h for radiolabeled parasite-binding experiments and 1×10^5 in 60 mm dishes for Giemsa staining before cholesterol depletion. Cholesterol replenishment was carried out on cells plated at a density of 2×10^5 on 35 mm culture dishes and grown for 24 h. These were first depleted of cholesterol using 10 mM MβCD in serum-free medium at 37 °C for 30 min. Cholesterol-depleted macrophages were replenished with cholesterol using cholesterol–MβCD complex. The complex was prepared by dissolving the required amounts of cholesterol (Sigma) and MβCD in half the required volume of deionized water at 80 °C with intermittent vortexing. After all the cholesterol was solubilized and the solution attained room temperature, equal volume of 2 × serum-free RPMI medium was added to yield a final concentration of 3.7 mM cholesterol and 37 mM MβCD (cholesterol:MβCD = 1:10 mol/mol ratio). The final concentration of cholesterol used was 1 mM. Increasing amounts of cholesterol was replenished into cholesterol-depleted cells when the treatment was carried out with 1 mM (indicates concentration of cholesterol) of the complex for increasing time periods. Cell monolayers were washed twice with PBS after each treatment. Cells were lysed in cold buffer (10 mM Tris, 5 mM EDTA, pH 7.4) and total cellular cholesterol was estimated in the lysates using the Amplex Red cholesterol assay kit (Molecular Probes) as described earlier [14]. Cholesterol values were normalized to protein levels estimated using the bicinchoninic acid (BCA) assay reagent kit (Pierce) as described previously [15].

2.4. Labeling Leishmania donovani AG83 promastigotes with tritium or FITC for binding studies

Parasites were metabolically radiolabeled with tritium as described earlier [16] with some modifications. Radiolabel incorporation was carried out at a density of 1×10^7 cells/2 ml of M-199 medium in the presence of 8 μCi/ml [^{3}H]thymidine (specific activity 89.4 Ci/mmol, New England Nuclear) at 22 °C for 3 h. FITC (Sigma) labeling of parasites was carried out as described previously [17], except that labeling was carried out at 37 °C in PBS.

2.5. Opsonization of Leishmania donovani AG83 promastigotes

Opsonization was carried out as described earlier [18] with a few modifications. Promastigotes were first radiolabeled with 20 μCi/ml [^{3}H]thymidine under conditions as described above. Radiolabeled promastigotes were opsonized with mouse serum at a density of 1×10^8 cells/ml at 37 °C for 25 min. Opsonized parasites were washed once with PBS and used directly.

2.6. Analysis of infected macrophages

Promastigotes were added onto macrophage monolayers at a parasite to macrophage ratio of 10:1 for indicated time periods, except for radiolabeled parasite-binding experiments with cholesterol-replenished cells where the ratio was 20:1 for a period of 3 h. The time-dependent radiolabeled parasite interaction with macrophages was monitored by allowing the infection to progress for 15, 30, and 90 min in RPMI-1640 medium at 37 °C. At the end of the incubation, monolayers were washed twice with

PBS to remove free parasites and placed on ice to loosen adherent cells from the plates. The monolayers were 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 different time periods. After infection, macrophages were gently scraped into PBS with 0.1% formaldehyde at 4 °C. The fluorescence from the FITC-labeled parasites associated with 5000–10,000 macrophages was analyzed with a Beckman Coulter ELITE ESP flow cytometer using the EXPO 32 software for data analysis. To assess the percentage of amastigotes in macrophages, the infection was allowed to proceed for 3 h before being stained with Giemsa. The number of amastigotes in macrophages was visually scored using a Leica microscope with a 100× oil objective.

2.7. Labeling of *Escherichia coli* with FITC

FITC labeling of *E. coli* was carried out as described previously [19] with some modifications. Briefly, *E. coli* DH5α cells were grown overnight under shaking conditions at 37 °C in Luria broth with 4 mg/ml FITC. Cells were pelleted down and washed five to six times with PBS to remove unbound stain. Cells were fixed with 4% paraformaldehyde at 4 °C for 1 h and washed three times with PBS to remove residual fixative. FITC-labeled cells were added to macrophages at a density of 1:200 (macrophage:bacteria ratio). Infection was allowed to proceed for 30 min at 37 °C. The macrophages were later washed extensively to remove unbound and/or floating bacteria followed by scraping the macrophages and washing them by spinning them down. The macrophages were then fixed with 4% paraformaldehyde before FACS analysis was carried out as described before.

3. Results

The objective of the present study was to determine the role of cholesterol in bringing about a productive leishmanial infection in mammalian host cells. The murine macrophage cell line J774A.1 was used as a host [11] to a virulent *L. donovani* strain AG83 (MHOM/IN/1983/AG83). Selective cholesterol depletion from the plasma membranes of the host macrophages was achieved by treatment with MβCD, a compound that specifically extracts cholesterol from the plasma membranes leaving other lipids intact [20,21] and disrupts lipid rafts [12]. The extent of leishmanial infection in control and cholesterol-depleted macrophages using MβCD was assessed at the levels of (i) parasite interaction with the host cell surface as monitored by ligand-binding assays using metabolically radiolabeled promastigotes, the extracellular form of the parasites and confirmed by flow cytometric analysis using fluorescently labeled promastigotes and (ii) the eventual presence of the intracellular amastigote form of the parasite in macrophages.

3.1. Concentration-dependent depletion of J774A.1 macrophage membrane cholesterol with MβCD

Acute depletion of cholesterol was achieved using MβCD which brought about a concentration-dependent specific reduction in total cellular cholesterol levels in J774A.1 macrophages as assessed using the Amplex Red cholesterol assay kit [14]. The assay is based on oxidation of cholesterol catalyzed by cholesterol oxidase which generates hydrogen peroxide. The hydrogen peroxide thus generated is made to react with peroxidase to release oxygen which oxidizes the otherwise non-fluorescent Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to a highly fluorescent compound (resorufin). Control experiments carried out with J774A.1 cell lysates in the absence of cholesterol oxidase but in presence of peroxidase did not produce any significant amount of the fluorescent end product (see Fig. 1A). This suggests that the assay is specific for cholesterol in this system and rules out any possible artifact. Moreover, this assay has been previously used for detection of cellular cholesterol by a number of investigators [22–25]. Acute cholesterol depletion of macrophages resulted in an ∼40% reduction of total cellular cholesterol when 10 mM MβCD was used for 30 min (Fig. 1B). The concentration of MβCD used for all subsequent experiments was 10 mM except in Fig. 4.

3.2. Cholesterol depletion of J774A.1 macrophages reduces extent and kinetics of *Leishmania* promastigotes interaction with the host

To analyze the effects of cholesterol depletion on the ability of *Leishmania* parasites to interact with host macrophages, we carried out ligand-binding assays with [^{3}H]thymidine-labeled promastigotes. Cholesterol-depleted macrophage cells, when used as hosts against the parasite, display a reduction in kinetics and extent of the host–parasite interaction/attachment compared to control cells. This is evident when infectivity was monitored till 90 min (Fig. 2A). Depletion of cholesterol resulted in ∼45% reduction in macrophage–parasite interaction when compared to control cells. These results were further confirmed by carrying out flow cytometric analysis of FITC-labeled promastigotes. Fluorescent derivatization of promastigotes with FITC has previously been used as a convenient method 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 [17]. Data in Fig. 2B reveal a time-dependent reduction in fluorescence associated with 10 mM MβCD-treated macrophages as compared to control macrophages when infection is allowed to continue for 90-min duration. These data support the earlier conclusion (from Fig. 2A) that there is a reduction in the ability of *Leishmania* promastigotes to interact with host

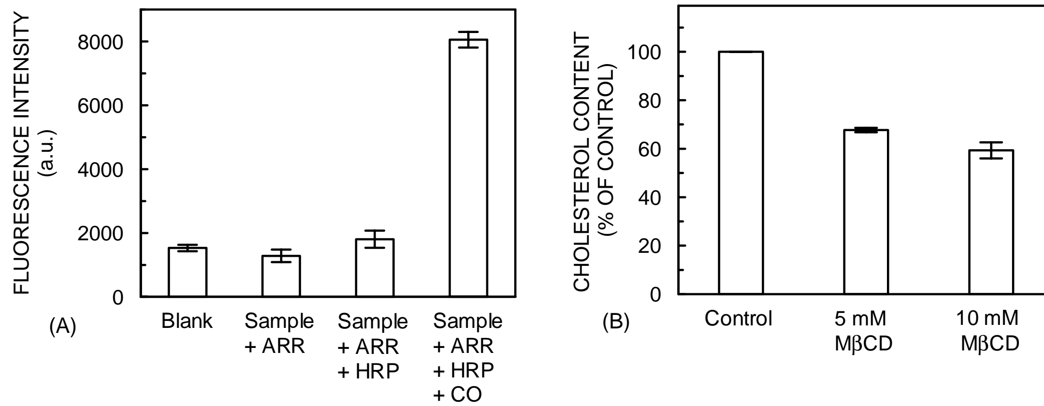

Fig. 1. Estimation of cholesterol in control and cholesterol-depleted macrophages. (A) Cholesterol estimation and specificity of the fluorimetric Amplex Red cholesterol assay for use with J774A.1 cell lysates. The graph shows that the assay is specific to report the cholesterol content since an increase in fluorescence intensity above the blank value is seen only upon addition of cholesterol oxidase (CO) enzyme to the assay. Incubation of J774A.1 cell lysate (Sample) with Amplex Red reagent (ARR) and horseradish peroxidase (HRP) enzyme in the absence of CO exhibits fluorescence intensity similar to the blank value. See Section 2 and Section 3.1 for other details. The data shown are the means ± S.E. of three independent experiments. (B) Total cellular cholesterol estimated using the Amplex Red cholesterol assay kit shows a concentration-dependent reduction upon treatment with MβCD. The data shown are the means ± S.E. of triplicate points from two independent experiments. See Section 2 for other details.

cells that are depleted of cholesterol. Moreover, as seen in Fig. 2B, these effects become pronounced as the time of infection is prolonged. We speculate that the differences in the time-dependent effect of cholesterol depletion on interaction/attachment of the parasite to macrophages seen between Fig. 2A and B, might arise on account of the use of FITC-labeled promastigotes (rather than radiolabeled promastigotes which are used for experiments shown in Fig. 2A) for FACS analysis (Fig. 2B). In spite of these differences, it is evident that cholesterol depletion of macrophages affects the ability of *Leishmania* promastigotes to interact with their host. Acute cholesterol depletion effects seen in such a relatively short time of exposure of parasites to macrophages might reflect a phenomenon occurring between the parasite and host cell surfaces, such as altering recognition or binding events, that would have otherwise led to a productive infection. These results (shown in Fig. 2A and B) therefore demonstrate that a loss in cholesterol content is associated with a corresponding loss of attachment and binding of the parasite to host macrophages.

3.3. Cholesterol depletion of J774A.1 macrophages does not affect the interaction of Escherichia coli DH5α strain of bacteria with the host

As a control, we monitored the effect of cholesterol depletion of macrophages on the uptake of another microbe, namely *E. coli* DH5α under similar conditions as that used to measure binding/attachment for *Leishmania* promastigotes. Flow cytometric analysis of control and cholesterol-depleted J774A.1 cells (Fig. 2C) infected with FITC-labeled *E. coli* for 30 min at 37 °C, thus measuring uptake of the bacteria as described in Ref. [19], did not show a reduction in binding/attachment unlike what was observed with *Leishmania* promastigotes (see Fig. 2A and B). In addition, these results are in agreement with a previous report [26] describing the lack of sensitivity of cholesterol depletion on the entry of radiolabeled *E. coli* and other bacteria, such as *S. typhimurium*, *Y. pseudotuberculosis*, and *L. casei*, into J774A.1 macrophages. Our results point to the specificity of cholesterol-dependent interaction between *Leishmania* promastigotes and the macrophage cell surface.

3.4. Cholesterol depletion does not affect the extent and kinetics of interaction of opsonized Leishmania donovani with the host

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, 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 [2]. To further understand the molecular mechanisms behind the reduced infectivity of *Leishmania* promastigotes in cholesterol-depleted macrophages, we carried out binding studies with radiolabeled promastigotes which were opsonized with mouse serum. These results (shown in Fig. 3) indicate a lack of sensitivity of cholesterol depletion on the uptake/binding of opsonized parasites into J774A.1 macrophages. Thus, cholesterol depletion of macrophages does not affect the route of entry undertaken by opsonized parasites.

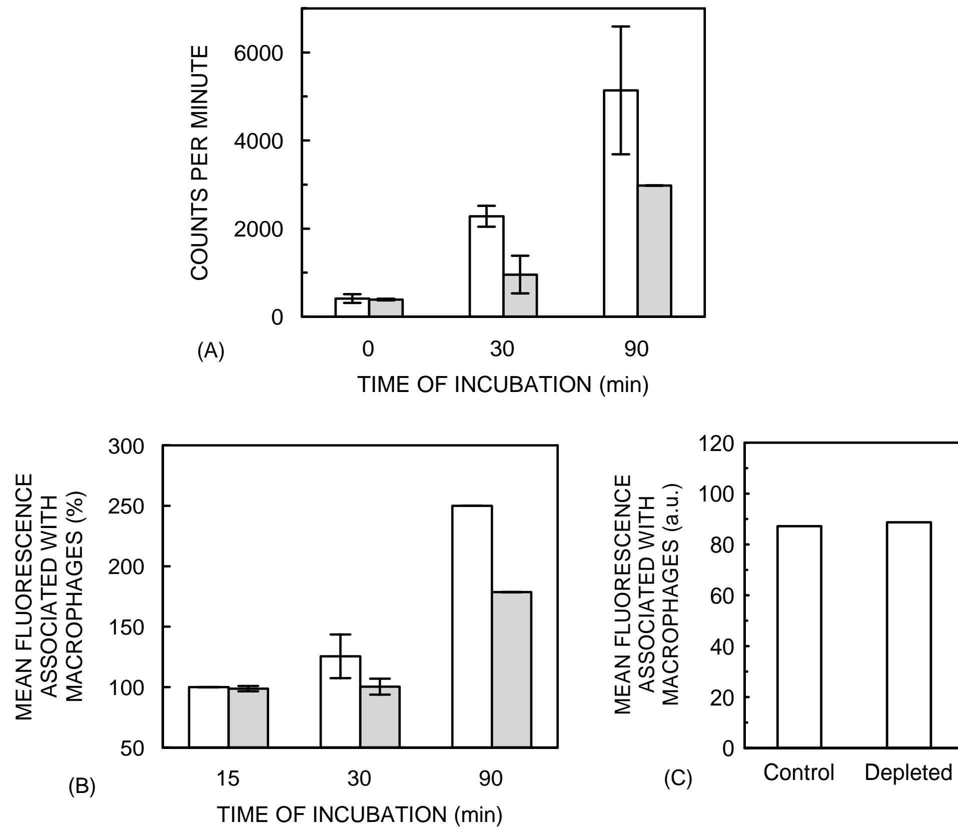

Fig. 2. Effect of cholesterol depletion on binding of *Leishmania* promastigotes and *Escherichia coli* DH5α cells to J774A.1 macrophages. (A) Effect of cholesterol depletion on binding kinetics of radiolabeled *Leishmania* promastigotes. Multiplicity of infection was maintained at 10:1 of parasite to macrophage. Comparison of binding of radiolabeled promastigotes between control (white bars) and cholesterol-depleted macrophages using 10 mM MβCD (gray bars) reveals a reduction in extent and kinetics of binding monitored up to 90-min duration. The data shown are means ± S.E. of three independent experiments. (B) Effect of cholesterol depletion on binding kinetics of FITC-labeled promastigotes to J774A.1 macrophages monitored by flow cytometry. FITC-labeled parasites were added onto macrophages at a ratio of 10:1. Values are normalized to the fluorescence associated with control macrophages after 15-min exposure to the parasite. Data are the mean ± S.D. of two independent experiments. The data reveal a time-dependent reduction in fluorescence associated with 10 mM MβCD-treated macrophages (gray bars) as compared to control macrophages (white bars). (C) Effect of 10 mM MβCD-mediated cholesterol depletion of J774A.1 cells on binding of FITC-labeled *E. coli* DH5α studied using flow cytometry. Infection was carried out for 30 min at 37 °C at a multiplicity of infection maintained at 200:1 of bacteria to macrophage. Representative data shown in the figure indicate a lack of sensitivity of binding of *E. coli* to macrophages depleted of cholesterol unlike the case seen with *Leishmania* promastigotes. See Section 2 for other details.

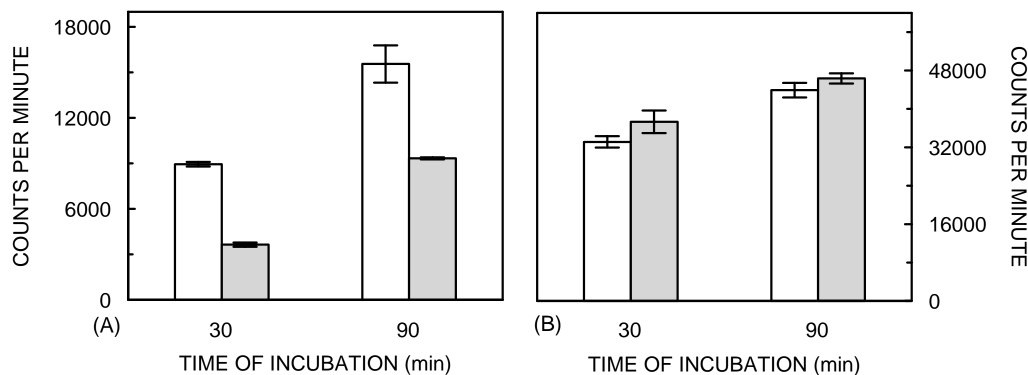

Fig. 3. Effect of cholesterol depletion on binding of opsonized radiolabeled *Leishmania* promastigotes. Opsonization of radiolabeled parasites was carried out as described in Section 2. Multiplicity of infection was maintained at 10:1 of parasite to macrophage. (A) Comparison of binding of radiolabeled promastigotes to control (white bars) and cholesterol-depleted macrophages using 10 mM MβCD (gray bars) reveals a reduction in extent and kinetics of binding monitored up to 90-min duration. (B) Comparison of binding of opsonized radiolabeled promastigotes between control (white bars) and cholesterol-depleted macrophages using 10 mM MβCD (gray bars) under similar conditions, as in panel A, reveal no change in extent and kinetics of binding monitored up to 90-min duration. The data shown are means ± S.E. of three independent experiments. See Section 2 for other details.

3.5. Reduction in extent and kinetics of parasite binding is associated with a reduction in intracellular load of parasites in cholesterol-depleted cells

The above results conclusively demonstrate that cholesterol depletion leads to a reduction in the ability of promastigotes to interact with and 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 cholesterol-depleted macrophages was determined visually after staining the infected macrophages with Giemsa. Treatment of macrophages with MβCD (5 and 10 mM) caused a concomitant reduction in the number of amastigotes present (compared to control cells) in the macrophages with only 50% amastigotes present when depletion was carried out with 10 mM MβCD (Fig. 4).

3.6. Restoration of parasite-binding ability to host cell surface upon replenishment of membrane cholesterol

The above results show that leishmanial infection in J774A.1 cells is reduced at the initial binding/attachment stage when the host plasma membrane is depleted of cholesterol and this leads to a reduction in the intracellular load of the parasite. In order to pinpoint whether these effects were specifically due to removal of cholesterol, we replenished the cholesterol content of cholesterol-depleted cells. Cholesterol replenishment was achieved by treating cells with a 1:10 (mol/mol) complex of cholesterol and MβCD, respectively, as described in Section 2. This treatment led to very efficient delivery of cholesterol back to the cells (Fig. 5B). Macrophages treated this way were infected with radiolabeled promastigotes to assess parasite-binding ability. Interestingly, the increase in cellular cholesterol content led to a concomitant increase in the ability of the parasite to attach to the macrophage cell surface (Fig. 5A). These results therefore confirm that the reduced ability of the parasite to bind to the macrophages depleted of cholesterol is a specific effect that can be reversed upon cholesterol replenishment.

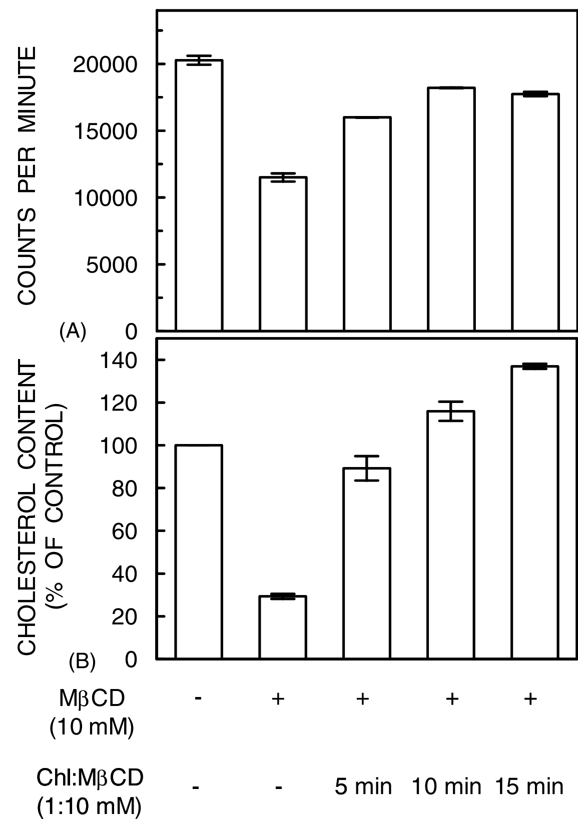

Fig. 5. Effect of replenishment of cholesterol into cholesterol-depleted J774A.1 macrophages on the ability of the radiolabeled parasite to bind to the macrophages. Macrophages were depleted of cholesterol using 10 mM MβCD. Cholesterol was replenished into these macrophages using cholesterol complexed with MβCD at a final concentration of 1 and 10 mM, respectively. Parasite to macrophage ratio was 20:1 and infection was carried out for 3 h. (A) Cholesterol replenishment into 10 mM MβCD-treated cells increasingly support radiolabeled parasite-binding ability. The extent of cholesterol replenishment upon treatment with cholesterol–MβCD complex for varying time periods (5, 10, and 15 min) is shown in panel B. The data shown are means ± S.E. of three independent experiments performed in duplicate. See Section 2 for other details.

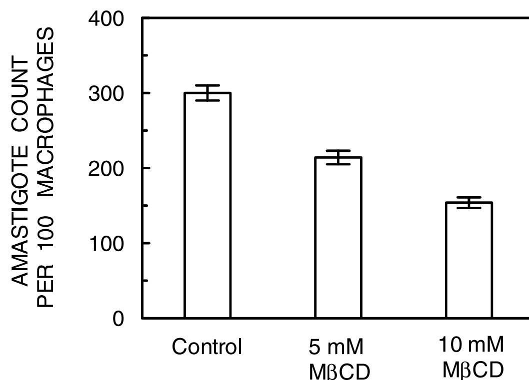

Fig. 4. Effect of cholesterol depletion on internalization of the parasite assessed by the amastigote count in infected J774A.1 macrophages. Macrophages depleted of cholesterol using 5 and 10 mM MβCD, exposed to parasites at multiplicity of infection of 10:1 for 3 h, show a reduction (nearly 50% in the case of 10 mM MβCD treatment) in the number of intracellular amastigotes as revealed by Giemsa staining. The data shown are the means ± S.E. of six independent experiments. See Section 2 for other details.

4. Discussion

Our results clearly show that plasma membrane cholesterol plays a crucial role in leishmanial infection, and cholesterol depletion from macrophages by MβCD results

in a significant reduction in the extent of leishmanial infection. We demonstrate this by ligand binding using metabolically radiolabeled promastigotes and also by flow cytometric analysis of fluorescently labeled promastigotes. This reduction in binding of the promastigotes to macrophages is accompanied by a concomitant reduction in the number of the intracellular amastigote form of the parasite. Importantly, the reduction in binding of the parasite to cholesterol-depleted macrophages can be reversed by replenishment of cholesterol thus reinforcing the specific requirement of cholesterol in the infection process.

The molecular mechanism of how cholesterol supports binding of the parasite to the macrophage cell surface requires further investigation. Interestingly, the effects of cholesterol depletion 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 [2]. 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 [13], galanin receptor [27], and the serotonin$_{1A}$ receptor (Pucadyil TJ, Chattopadhyay A, unpublished observations), has been previously demonstrated. These results show that cholesterol depletion may lead to perturbation of receptor–cholesterol interactions leading to loss of receptor function. The reduction in infectivity of the parasite accompanied with cholesterol depletion could be due to such alterations in interactions of one or more of the many receptors that have been proposed to have a role in the attachment and internalization of the parasite [2].

Our results conclusively indicate that cholesterol depletion of macrophages affects the binding/attachment of leishmanial parasites. In light of the essential role of cholesterol to maintain membrane integrity, modulate membrane receptor function and ensure cell viability [4,28], the issue of specificity of the effects of cholesterol depletion observed in this study deserves comment. We observed that cholesterol in J774A.1 macrophage membrane is necessary for binding/attachment of *Leishmania* promastigotes but not for *E. coli* DH5α strain. This indicates that interaction between the parasites and the macrophage cell surface is cholesterol-dependent and specific for *Leishmania* under the present conditions. Moreover, the observation that the attachment/binding of opsonized *Leishmania* parasites is not affected when macrophages are depleted of cholesterol, serves as an additional control and further stringently defines the specificity of interaction of one form of the parasite (non-opsonized) with the host cell surface. More importantly, the loss in binding/attachment associated with cholesterol depletion of the macrophage cell surface is a reversible phenomenon and is dependent only on membrane cholesterol content. This clearly signifies that cholesterol depletion and the associated reduction in leishmanial infectivity is not due to a general disruption in cell membrane integrity and cell viability, but results from a specific disruption of a cholesterol-mediated entry/attachment process employed by the parasite.

The role of lipid rafts in general and cholesterol in particular is being increasingly recognized in signal transduction [9,29] and entry of pathogens to infect host cells [10]. The involvement of rafts has earlier been shown to control the infection of HIV type 1 [30,31], entry and internalization of mycobacteria [18,26] and *Brucella suis* [32] into macrophages, and infection of erythrocytes by the malaria parasite *Plasmodium falciparum* [24,33]. Our results show that this could be true for entry of *L. donovani* and its ability to infect host macrophages.

Cholesterol-mediated entry of *Leishmania* into host cells and reduction in infection may lead to novel therapeutic strategies against leishmaniasis. Cyclodextrin-like molecules have earlier been shown to be pharmacologically important in treating unstable atherosclerotic plaques due to their ability to remove cholesterol from macrophage foam cells in vitro [34]. In addition, β-cyclodextrin, a compound analogous to MβCD and which selectively extracts cholesterol from plasma membranes, has been shown to cause hypolipidemic effects in genetically hypercholesterolimic rats when fed orally [35]. Very recently, topical application of β-cyclodextrin has been shown to block the transmission of cell-associated HIV-1 [36]. This is based on earlier observations on the involvement of cholesterol in the infection of HIV type 1 [30,31]. Thus, the feasibility of administrating cyclodextrin-like molecules in vivo has already been established. Such administration of MβCD may block or significantly reduce leishmanial infection and further studies in the preclinical and clinical settings are needed to fully explore this issue.

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