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Down-regulation of mannose receptors on macrophages after infection with *Leishmania donovani*

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Macrophages express a mannose-specific endocytosis receptor that binds and internalizes mannose-terminated glycoproteins. Infection of mouse peritoneal macrophages with *Leishmania donovani* resulted in a decrease in mannose-receptor activity. With ^{125}I-labelled β-glucuronidase as ligand, a 2-fold decrease in uptake rate was observed in infected cells, with no change in K_{uptake}. Cell-surface binding of ^{125}I-mannose-BSA was diminished 2.5-fold after infection. The decrease in ligand binding appeared to be due to a decrease in the number of sites, with no change in affinity. Elimination of parasites from infected cells by treatment with neoglycoprotein-conjugated methotrexate resulted in an increase in receptor number. Cycloheximide suppressed the drug-treatment-mediated rise in receptor number in infected macrophages. A decrease in receptor activity was also observed in liver Kupffer cells isolated from parasite-infected mice. Binding of ligand by another carbohydrate receptor, the mannose 6-phosphate receptor, was not altered by infection. Phagocytosis of yeast cells was also not altered. These results suggest that mannose receptor synthesis in macrophages is specifically suppressed after infection with *Leishmania* parasites.

INTRODUCTION

Mammalian macrophages are known to express cell-surface receptors which specifically bind and internalize mannose-terminated glycoproteins [1,2]. The ligand glycoproteins are degraded within lysosomes, whereas the receptor apparently functions repeatedly without being degraded [3,4]. The receptor binds ligand at the cell surface by a Ca^{+2}-sensitive interaction; the receptor–ligand complexes are then clustered in coated pits and rapidly internalized into coated vesicles [5]. These become uncoated smooth vesicles and subsequently, in the acidic environment of a series of prelysosomal vesicles and tubules referred to as CURL (compartment for the uncoupling of receptor and ligand), the receptor and ligand part company and the ligand is dispatched to lysosomes, whereas the receptor recycles back to the surface [6]. In our earlier studies [7,8], we investigated whether this high-affinity sugar-specific glycoprotein uptake system of macrophages can be used for macrophage-specific drug targeting, using leishmaniasis as the model disease. Visceral leishmaniasis is a widespread parasitic disease throughout much of the Third World. The disease is caused by the protozoan *Leishmania donovani*, an obligate intracellular parasite in man that resides and multiplies within macrophages of the reticuloendothelial system [9]. The exclusive presence of mannose receptors on the macrophage surface together with the obligatory localization of *Leishmania* parasites within macrophage lysosomes formed the basis of our drug delivery using the neoglycoprotein mannosyl-BSA (Man-BSA) as the carrier for methotrexate. The neoglycoprotein–drug conjugate was found to be highly effective in eliminating the parasite burden in both the macrophage model *in vitro* and the animal model of visceral leishmaniasis *in vivo* [7,8].

Macrophage mannose receptor expression is known to be regulated by a variety of agents. The receptor is not present on freshly isolated peripheral blood monocytes or bone marrow monocytic precursor cells before cell culture, but starts appearing after 3 days in culture [10]. Receptor expression is increased by treatment with dexamethasone [11] and vitamin D [12], whereas activation *in vivo* by *Bacillus* Calmette–Guerin [13,14] or treatment with γ-interferon [15], swainsonine [16] or H_2O_2 [17] decrease expression. Since infection of macrophages by *Leishmania* parasites is also an activation process, and since our drug delivery method is based on the exploitation of surface mannose receptors, it was thought worthwhile to study the status of mannose receptors on the macrophage surface after *Leishmania* infection.

MATERIALS AND METHODS

Parasites

Leishmania donovani strain UR6 (MHOM/IN/1978/UR6) and strain AG83 (MHOM/IN/1983/AG83) were isolated from Indian patients with kala-azar [18,19]. UR6 was maintained in modified Ray's medium [18] and AG83 was maintained in BALB/c mice by intravenous passage every 6 weeks.

Cell culture

Peritoneal macrophages. Macrophages were collected by peritoneal lavage from mice (BALB/c, 20–25 g) given intraperitoneal injections of 0.5 ml of thioglycollate broth 5 days before harvest, and were cultured as described earlier for rats [20]. The composition of the macrophage culture medium (α-10) as described earlier [20] is α-minimal essential medium (MEM) (Gibco 430-1900) plus $NaHCO_3$ (2.2 g/l), 10% (v/v) fetal bovine serum (FBS), 100 units of penicillin/ml and 100 μg of streptomycin/ml.

Liver Kupffer cells. The cells were isolated from normal and *Leishmania*-infected BALB/c mice (20–25 g) by perfusion with collagenase according to the modified method of Berry & Friend [21] and Munthe-Kaas & Seglen [22], as described in [23]. The cells obtained from the metrizamide gradient were suspended in maintenance culture on Petri dishes according to the method of Dijkstra *et al.* [24]. The culture medium consisted of Dulbecco's medium supplemented with 10 mM-$NaHCO_3$, 20 mM-Hepes, penicillin (100 units/ml), streptomycin (100 μg/ml) and 20% heat-inactivated FBS (pH 7.4). The cells [(1.0–2.0) $\times$ 10^6 cells per

Abbreviations used: MEM, minimal essential medium; Man-BSA, mannosyl-BSA; PBS, phosphate-buffered saline 20 mM-phosphate/150 mM-NaCl, pH 7.0; FBS, fetal bovine serum.

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35 mm dish] were used for binding experiments after 2–3 days in culture.

Infection of macrophages

Promastigotes were used to infect cultures of adherent macrophages at a ratio of 10 parasites per macrophage. Infection was permitted to proceed for 4 h at 37 °C, after which all extracellular organisms that had failed to parasitize the macrophages were eliminated by washing. In most instances, 90% of the macrophages were found to be infected. Infected macrophages were incubated in macrophage culture medium for the indicated periods before carrying out binding and uptake studies.

Animal infection

L. donovani strain AG83 was used to infect BALB/c mice (15–20 g) through the tail vein (2×10^6 parasites per mouse). At 45 days after infection, the spleen weight had increased from 40.8 ± 8.5 mg in normal animals to 378.7 ± 52.3 mg in infected animals. Multiple spleen impression smears were prepared and checked for parasites using Giemsa stain. Kupffer cells were isolated from 45-day-infected animals.

Binding assay with ^{125}I-Man-BSA

Man-BSA was prepared according to the method described earlier [25], and was iodinated with $Na^{125}I$ by the chloramine T method [26] to a specific radioactivity of $(3–5) \times 10^6$ c.p.m./μg. Binding studies were performed with peritoneal macrophages and liver Kupffer cells in α-MEM plus 10% (v/v) FBS, buffered with 20 mM-Mops adjusted to pH 7.0. Cells were first scraped with a rubber policeman and were gently suspended in the binding medium, washed twice with the medium, cooled to 4 °C and incubated for 120 min with radioiodinated ligands in a total volume of 0.5 ml. Non-specific binding was determined in the presence of a 100-fold excess of unlabelled ligand or mannan (2 mg/ml), and was $< 15\%$ of the total binding. After the 4 °C incubation, the medium was removed and the cells were washed four times with Ca^{2+}/Mg^{2+}-containing phosphate-buffered saline (PBS). The cell pellet was assayed for radioactivity in a γ-radiation counter and the protein was estimated by the Lowry procedure [27].

Uptake and degradation studies

For time course studies, ^{125}I-Man-BSA was added at a concentration of 1 μg/ml (10^6 c.p.m./μg). To determine non-specific uptake, companion wells received 1 mg of mannan/ml. At various times after addition of ligand, the medium was removed (for degradation studies) and the cells were washed twice with PBS. The cell monolayers were solubilized in 0.1% Triton X-100 and counted for radioactivity by means of a γ-radiation counter. Ligand degradation was measured by determining the radioactivity liberated from Man-BSA in the culture medium after precipitation with trichloroacetic acid.

Phagocytosis assay

Phagocytosis of stained yeast by macrophages was measured by the method of Kaminski *et al.* [28]. Briefly, *Saccharomyces fragelis* (A.T.C.C. 10022) was stained with Congo Red (3 ml of a 0.87% (w/v) solution of Congo Red in PBS was added to 1.5 g of yeast] and the concentration was adjusted to 4×10^7 yeast cells/ml. Macrophages (3×10^6 cells/dish) were cultured in 35 mm Petri dishes and 6×10^7 stained yeast cells per dish were added for 1 h. Cells were then washed with PBS, removed from the dishes by treatment with trypsin/EDTA and stored overnight to solubilize the phagocytes, and the absorbance was then read in a spectrophotometer at 510 nm against a macrophage control.

Binding of mannose-phosphate-containing ligands by peritoneal macrophages

Both the mannose receptor and the mannose phosphate receptor on macrophages could be measured by using ^{125}I-α-mannosidase as ligand [29]. This ligand was added in a total volume of 0.5 ml of α-MEM plus 10% FBS buffered with 20 mM-Mops, pH 7.0, containing 1 μg of ligand. The cells were then washed and solubilized in 0.1% Triton X-100, and the cell-associated c.p.m. were quantified by γ-radiation counting.

RESULTS

Inhibition of mannose receptor activity in mouse peritoneal macrophages infected with *L. donovani*

β-Glucuronidase is known to be taken up efficiently by cultured macrophages via the mannose receptor [11]. Uptake is linear with time and saturable with increasing ligand concentration. Fig. 1 shows the effect of increasing concentrations of added ^{125}I-β-glucuronidase on uptake at 37 °C in control and *L. donovani*-infected cells. Macrophages were infected as described in the Materials and methods section and incubated in culture medium for 20 h before carrying out uptake studies. In both cases, uptake was saturable (at 150 μg/ml). However, uptake in infected cells was only approx. half that in control cells. A double reciprocal plot of the uptake data (Fig. 1, inset) demonstrate that K_{uptake} was unaffected by *Leishmania* infection ($K_{uptake} = 2.55 \times 10^{-7}$ M),

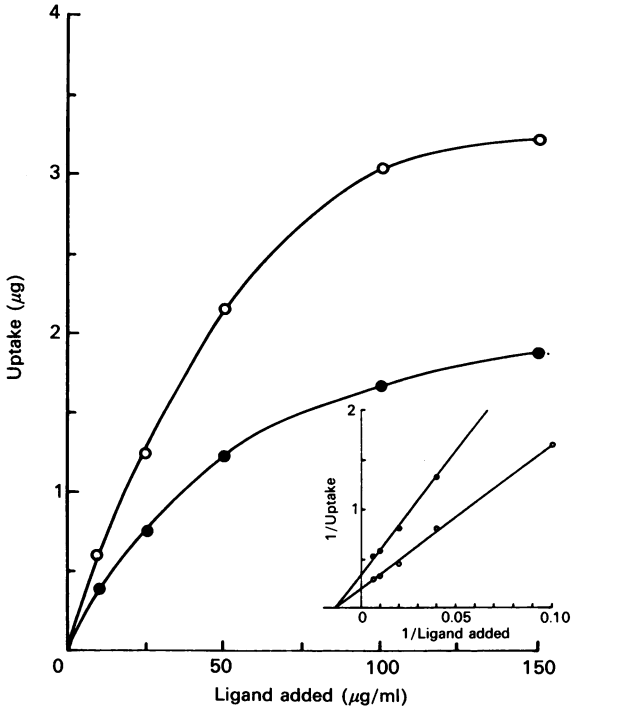

Fig. 1. Effect of *L. donovani* infection on uptake of ^{125}I-β-glucuronidase by macrophages

Mouse peritoneal macrophages were infected with *L. donovani* for 4 h as described in the Materials and methods section, excess parasites were washed off and the infected cells were cultured for a further 48 h. Bovine liver β-glucuronidase was iodinated by the chloramine T method [26]. Uptake of ^{125}I-β-glucuronidase at increasing concentrations was measured in both control (○) and infected (●) macrophages. The inset shows a double-reciprocal plot of the data, yielding a K_{uptake} of 2.55×10^{-7} M for both control and infected cells.

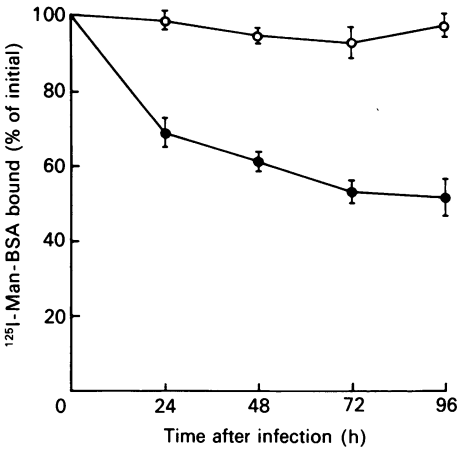

Fig. 2. Macrophage mannose receptor activity as a function of time after infection with *L. donovani*

Macrophages were infected with *L. donovani* for 4 h, and then infected cells were cultured for up to 4 days. Receptor binding assays were performed on infected cells at indicated time intervals as described in the Materials and methods section, using ^{125}I-Man-BSA as ligand (2 μg/ml; 10^6 c.p.m./μg) in the presence and the absence of mannan (2 mg/ml). Data represent means ± S.D. of three independent determinations. ○, Control; ●, infected.

whereas the rate of uptake in infected cells was half that in controls.

Effect of infection on the expression of mannose receptors by peritoneal macrophages

When peritoneal macrophages were infected with *L. donovani* for 4 h and kept for various intervals of time, the expression of mannose receptors was found to be suppressed in a time-dependent manner. Receptor expression was determined by binding experiments using ^{125}I-Man-BSA as ligand in all subsequent experiments, as this ligand is prepared synthetically and is therefore more chemically defined than β-glucuronidase. Binding was determined at 4 °C, where uptake is essentially zero. Infection for 24 h resulted in a loss of about 30% of binding activity (Fig. 2). Infection for 4 days resulted in a loss of approx. 50% of original binding activities. The possibility that the decreased expression of mannose receptors is a direct consequence of cell death after infection was ruled out by evaluation of the number of attached cells, viability (Trypan Blue exclusion) and release of lactate dehydrogenase from cells.

Decreased ^{125}I-Man-BSA binding to macrophages after infection with *L. donovani*

To determine the basis for the decreased binding activity of infected macrophages for Man-BSA, Scatchard analysis of the binding data with various concentrations of ^{125}I-Man-BSA from infected and control cultures was performed. Saturation of binding in infected cells occurs with approx. 40% of the ligand bound, as seen with control cells (Fig. 3). The plot of the binding data demonstrates that the parasite infection caused a decrease in the total number of receptors available rather than in the binding affinity (Fig. 3, inset). To further ascertain the decreased binding after infection, the effects of known regulators of mannose receptors, such as dexamethasone, H_2O_2, swainsonine and glucose/glucose oxidase, on the binding of ^{125}I-Man-BSA by peritoneal macrophages was examined. As shown in Table 1, except for dexamethasone, which increased receptor expression, all other agents caused a down-regulation of receptor activity.

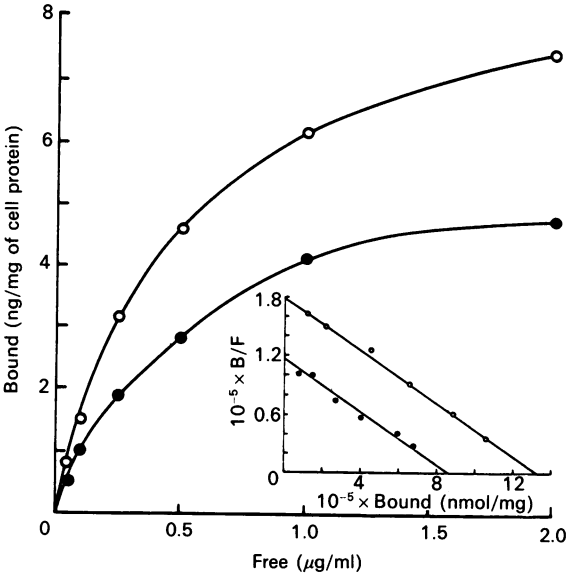

Fig. 3. Effect of *L. donovani* infection on binding of ^{125}I-Man-BSA

A 48 h culture of infected macrophages was used for binding along with a parallel control macrophage culture. Binding was determined on ice (4 °C) with $(3–5) \times 10^5$ cells in 0.5 ml of α-MEM containing 20 mM-Mops, pH 7, and supplemented with 10% FBS. ^{125}I-Man-BSA (3.5×10^6 c.p.m./μg) in medium was added to initiate the binding reaction as described in the Materials and methods section. Non-specific binding (i.e. binding in the presence of unlabelled mannan, 2 mg/ml) was subtracted from the total. A Scatchard plot (inset) was constructed from the specific binding data, yielding K_d values of 7.44×10^{-9} M and 7.41×10^{-9} M, and $B_{max.}$ values of 13.25 nmol/mg of cell protein and 8.60 nmol/mg of cell protein for control (○) and infected (●) cells respectively.

Table 1. Modulation of macrophage mannose receptor activity by various agents

Mouse peritoneal macrophages were treated with various agents as indicated. In case of *L. donovani* treatment, macrophages were infected with the parasite for 4 h; excess parasites were then washed off and the cells were cultured in fresh medium for 48 h. Receptor binding assays were performed as described in the Materials and methods section using ^{125}I-Man-BSA as ligand (2 μg/ml; 10^6 c.p.m./μg). All data are corrected for non-specific binding and are represented as mean ± S.D. of three independent determinations.

Agents	Treatment period (h)	^{125}I-Man-BSA binding (c.p.m./mg of cell protein)	^{125}I-Man-BSA binding (% of control)
None	—	7608±655	100
H_2O_2 (1 mM)	0.5	3672±417	48
Glucose (10 mM)/ glucose oxidase (1000 munits)	0.5	4144±452	54
Dexamethasone (0.25 μM)	16	14433±975	190
Swainsonine (0.3 μM)	2	2906±373	38
L. donovani	48	4615±483	61

Effect of *Leishmania* infection on Man-BSA degradation

Fig. 4 shows the effect of infection by *Leishmania* parasites on the degradation of Man-BSA by macrophages. Since the infection caused a decreased binding of Man-BSA by macrophages, an equivalent amount of bound radioactivity was taken for degradation studies in the case of both normal and infected cells. Although in both normal and infected cells the release of acid-

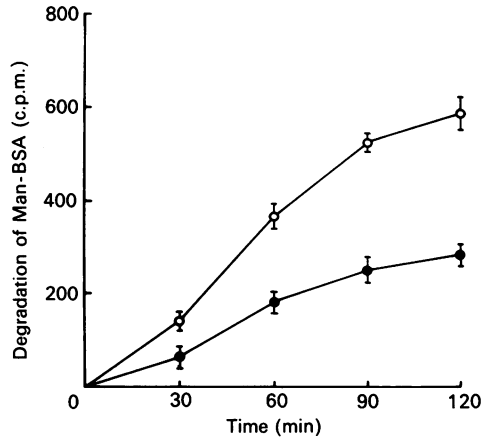

Fig. 4. Degradation of 37 °C of ^{125}I-Man-BSA previously bound by normal and infected macrophages

Both normal (○) and infected (●) macrophages were incubated with ^{125}I-Man-BSA (2 μg/ml) for 1 h at 4 °C. Cells were then washed and normal and infected cells containing equivalent amounts of bound radioactivity were reincubated with fresh medium at 37 °C. At different time points the radioactivity released from the cells as trichloroacetic acid (20%)-soluble molecules in the culture supernatant fraction was assessed. Data represent means ± S.D. of three independent determinations.

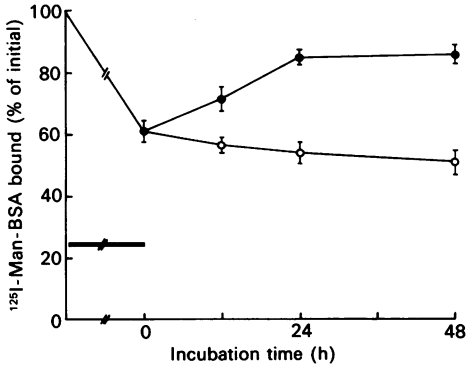

Fig. 5. Recovery of ^{125}I-Man-BSA binding activity after the removal of intracellular parasites

Mouse peritoneal macrophages were infected with *L. donovani* for 4 h and cultured for 48 h (bar). Cells were then treated with neoglycoprotein-conjugated methotrexate (1 μg/ml) for 3 h as described earlier [7]. Cells were washed free of the drug and resuspended in fresh medium. After various times at 37 °C, ^{125}I-Man-BSA binding activity was determined. Data presented are means ± S.D. from three independent determinations. ○, Control; ●, drug-treated.

soluble radioactivity almost reached completion within 120 min, there was an almost 50% decrease in the appearance of degraded products in the case of infected cells.

Recovery of receptor activity after elimination of parasites

The loss of mannose receptor activity observed after infection by *Leishmania* parasites is followed by recovery of receptor activity after elimination of parasites from macrophages (Fig. 5). Removal of parasites from macrophages was brought about by treatment with 1 μg of methotrexate/ml conjugated with Man-BSA for 3 h as described previously [7]. Drug treatment caused a recovery of almost 65% of the inhibited receptor activity. To ascertain whether reappearance of receptor activity required *de novo* synthesis of receptor molecules, the effects of cycloheximide on receptor recovery were examined. Drug-treated infected macrophages were further cultured in α-MEM with 10% FBS in the presence of various concentrations of cycloheximide at 37 °C for 12 h. The result (Table 2) showed that 0.5 μg of cycloheximide/ml blocked almost 40% of ^{125}I-Man-BSA binding

Table 2. Effect of cycloheximide on the recovery of ^{125}I-Man-BSA binding activity

L. donovani-infected macrophages were treated with neoglycoprotein-conjugated methotrexate (1 μg/ml) for 3 h as described in [7]. This was shown to eliminate 85% of intracellular parasites. Cells were then washed free of the drug and replenished with fresh culture medium in the presence of various concentrations of cycloheximide at 37 °C for 12 h. Receptor binding assays were performed as described in the Materials and methods section. All data are corrected for non-specific binding and are means ± S.D. of three independent determinations.

Cycloheximide (μg/ml)	^{125}I-Man-BSA bound (c.p.m./mg of cell protein)
0	6327 ± 743
0.005	6646 ± 687
0.05	5892 ± 623
0.5	2648 ± 342
5	368 ± 28
50	216 ± 16

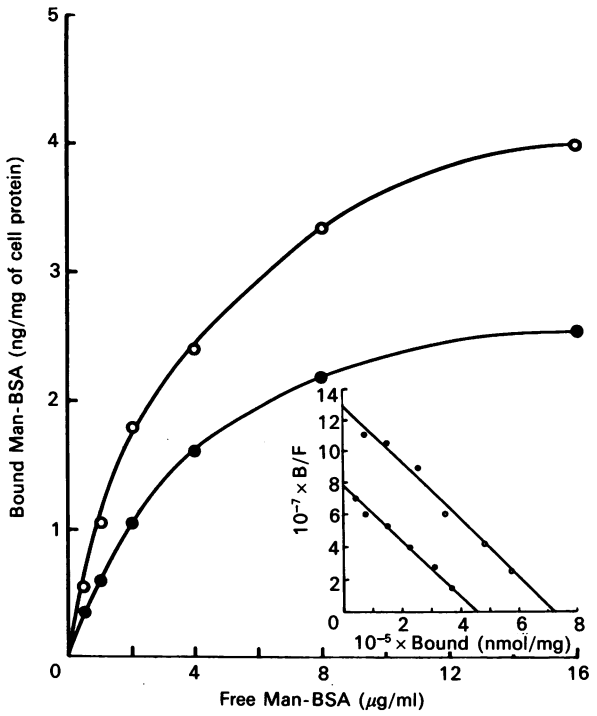

Fig. 6. Status of mannose receptor activity on liver Kupffer cells isolated from *L. donovani*-infected BALB/c mice

Kupffer cells were isolated from livers of both normal and *L. donovani*-infected BALB/c mice (45 day infection) and cultured as described in the Materials and methods section. Receptor binding assays were performed with increasing concentrations of ^{125}I-Man-BSA at 4 °C, as described in the legend to Fig. 3. The inset shows a Scatchard plot of the data, yielding K_d values of 5.6×10^{-8} M and 5.8×10^{-8} M, and $B_{max.}$ values of 7.20 nmol/mg and 4.55 nmol/mg of cell protein respectively for control (○) and infected (●) cells.

Table 3. Effect of *L. donovani* infection on other macrophage functions

Both normal and *L. donovani*-infected (48 h culture) peritoneal macrophages were taken to evaluate receptor activity using ^{125}I-α-mannosidase as ligand (2 μg/ml; 1.5×10^6 c.p.m./μg), according to [17]. Wells for determining binding via the mannose phosphate receptor received ligand plus 10 mM-mannose 6-phosphate; wells for determining mannose receptor activity received ligand plus mannan (2 mg/ml). The assay for phagocytosis is described in the Materials and methods section, and the data are expressed as numbers of yeast cells per 100 macrophages. Values in parentheses indicate uptake in infected cells as a percentage of control values.

Macrophage function	Uptake (c.p.m./mg of cell protein)	
	Normal	Infected
Mannose receptor activity	5248±711	3108±432 (59)
Mannose phosphate receptor activity	7956±837	7331±812 (92)
Phagocytosis	335±42	282±37 (84)

activity relative to that of drug-treated cells in the absence of cycloheximide. The viability of macrophages incubated with 0.5 μg of cycloheximide/ml was greater than 95%, as judged by Trypan Blue exclusion. In contrast, about 40% and 10% of cells were detached from plates at cycloheximide concentrations of 50 μg/ml and 5 μg/ml respectively.

Receptor status in liver Kupffer cells

In order to assess the receptor status *in vivo*, mannose receptor activity was also determined in liver Kupffer cells isolated from both normal and *L. donovani*-infected mice. Scatchard analysis of the specific binding of ^{125}I-Man-BSA measured at 4 °C revealed that parasite infection decreased the number of receptors by almost 40% in Kupffer cells compared with controls (Fig. 6). In this case also the affinity of the receptor was unaltered after infection. A 10-fold lower affinity of receptors on freshly isolated Kupffer cells compared with cultured peritoneal macrophages may be attributed to the isolation procedure, which results in enriched Kupffer cell preparation.

Effect of infection on other macrophage functions

To study the effect of parasite infection on other macrophage functions, binding of ligand by another macrophage receptor (the mannose 6-phosphate receptor) and phagocytosis of *S. fragelis* were measured [17]. Infection of peritoneal macrophages by *L. donovani* for 4 days decreased mannose-receptor-mediated uptake of ^{125}I-α-mannosidase to 50% of control levels, whereas it had very little effect on mannose-6-phosphate-specific recognition (Table 3). The effect of *L. donovani* infection on phagocytosis was also examined. Phagocytosis of *S. fragelis* by infected macrophages was decreased by only 16% compared with that by control cells (Table 3).

DISCUSSION

The present study demonstrates that infection of macrophages by *L. donovani* resulted in a decreased activity of the mannose receptor, which mediates uptake of mannose-containing glycoconjugates. This decreased receptor activity was found *in vitro*, i.e. in cultured peritoneal macrophages infected with parasites, as well as *in vivo*, i.e. in liver Kupffer cells isolated from parasite-infected mice. Following infection, both uptake and binding of mannose-containing ligands were inhibited. Scatchard analysis of the binding data shows no change in the affinity of the receptor for the ligand. However, a decrease in surface receptors to approximately one-half of the control level occurs following infection. These results suggest that parasite infection does cause receptors to become inaccessible for interaction with ligand. This is in contrast with the earlier observation by Rabinovitch *et al.* [30,31] that the uptake of mannose-terminated ligands was similar in infected and non-infected bone-marrow-derived macrophages. It is not clear at this stage whether this is due to differences in strains of parasite, as they used *Leishmania mexicana amazonesis*, which causes cutaneous leishmaniasis.

The decrease in receptor number appears to have a direct correlation with the number of amastigotes (host form of the parasite) within macrophages, for the following reasons. Receptor activity is diminished in infected cells with the progression of time after infection. Amastigote number is also known to increase with time of culture of cells after infection [18]. Moreover, elimination of amastigotes from infected macrophages by drug treatment resulted in the reappearance of receptor activity. Cycloheximide suppressed the drug-treatment-mediated rise in receptor number in infected macrophages, whereas cycloheximide alone had little effect on receptor activity. This suggests *de novo* synthesis of receptor molecules. Since the macrophage mannose receptor is a recycling receptor and the ligand is dispatched to lysosomes for degradation after dissociation from receptor–ligand complex, degradation of ligand was studied in both normal and infected cells. Degradation was inhibited in infected cells, suggesting a block of transfer of ligand to the compartment where degradation of ligand occurs and/or a prevention of degradation within this compartment after infection.

Other functions of macrophages, such as binding of another carbohydrate receptor (the mannose phosphate receptor) and phagocytosis of yeast cells, were tested after *Leishmania* infection. Although lysosomal enzymes are suppressed after infection as observed earlier [18], modulation appears to be fairly specific for the mannose receptor. Binding of ligand by the mannose phosphate receptor as well as phagocytosis of *S. fragelis* were not changed, thereby suggesting that these functions were minimally altered by the infection.

Several interesting possibilities exist regarding the receptor down-regulation which deserve further investigation, e.g. competition by parasite-released factors with ^{125}I-Man-BSA for its receptor, a decrease in receptor synthesis, impairment of transport of newly synthesized receptor to the plasma membrane, and down-regulation of receptor by a direct effect of parasite infection (e.g. formation of giant intracellular lysosomes) or by indirect effects (e.g. mediation by cytokines stimulated by parasite internalization). The question of whether the observed effect is due to decreased receptor synthesis or interference with the transport of receptors to the plasma membrane, or both, may be answered by the use of specific antibodies and cDNA probes for the mannose receptor. Down-regulation of the mannose receptor might also result from accelerated receptor degradation rather than from decreased synthesis. This possibility is not ruled out by the cycloheximide experiment or by the decreased degradation of the ligand. Accelerated receptor degradation upon infection would lead to a lower steady-state level, and the recovery after treatment would be affected by cycloheximide as observed.

Our earlier work on neoglycoprotein-mediated drug delivery in visceral leishmaniasis was based on expression of the mannose receptor on the macrophage surface. However, the present work suggests a decrease in receptor number after *Leishmania* infection, and therefore maximal targeting efficiency might not have been achieved in our previous studies. Since dexamethasone, an anti-inflammatory corticoid, up-regulates mannose receptor expres-

sion, it would be of interest to study drug delivery experiments in conjunction with dexamethasone to achieve better targeting efficiency.

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