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Studies on the effect of the anti-phagocytic agent cytochalasin B on *Leishmania*-macrophage interaction

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Summary

Mouse peritoneal exudate cells were cultured on coverslips in Eagle's Basal Essential Medium. The adhering cells were infected with promastigotes of three different species of *Leishmania.* After 8 h incubation, the macrophages were fixed and stained, and a total of one hundred cells were counted. The rates of infection of macrophages were respectively 53.5 ± 5% for *L. enriettii,* 52.3 ± 5% for *L. donovani* and 11.7 ± 2% for *L. tropica.* When cytochalasin B at concentrations of 2.5, 5 and 10 μg/ml and *Leishmania* promastigotes were added to the adhering cells at the same time, the drug did not have any effect on the uptake of the organisms by the macrophages. However, when the cells were treated for a 2-h period with the drug and then were infected with the promastigotes, only 1–2% of the cells were infected. On the other hand, when cytochalasin B-treated cells which had lost their phagocytic ability were washed and then were infected with the promastigotes, some degree of cellular infection was observed. It was concluded that infection of mouse p.e.c. by three different species of *Leishmania* which were used in our study was by phagocytosis rather than active penetration of the organisms into the cells. It was also of interest to note that although our outbred strain of mice gets infected easily with *L. tropica,* the p.e.c. of these animals phagocytosed *L. tropica* with least efficiency in comparison with *L. donovani* and *L. enriettii.*

Key words: Leishmania donovani; L. tropica; L. enriettii; macrophage; in vitro; mouse; peritoneal exudate cells.

Introduction

Leishmania organisms are parasites of mononuclear phagocytic cells in mammalian hosts. These organisms occur as amastigotes in vertebrate hosts

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and as promastigotes in invertebrates and cultures (Hoare and Wallace, 1966). There are controversial reports on the mechanism by which *Leishmania* promastigotes gain access to the host cells (Alexander, 1975; Lewis, 1974). It is not clear whether the organisms are engulfed by the cells or actively penetrate the host cell.

Previous studies by Lainson and Strangeways-Dixon (1963) have shown that *Leishmania mexicana* may exist inside epidermal pigment cells and cartilage cells which are not usually phagocytic; similarly it has been demonstrated (Bradley et al., 1973) that there are forms of *L. donovani* within non-phagocytic parenchymal cells of the liver of man, hamster and mouse. Recently, Alexander (1975) reported that *L. mexicana* promastigotes enter mouse macrophages by being phagocytosed. On the other hand, Lewis (1974) concluded that in vitro infection of dog sarcoma cells by *L. mexicana* is by "induced phagocytosis". There are also reports that indicate the possibility of an invasion of tissue by trypanosomatid parasites which are closely related to *Leishmania* (Sooksri and Inoki, 1972; Alexander, 1975). Vischer and Suter (1954) have proposed that infection of macrophages by the protozoon *Toxoplasma* is by normal phagocytosis but that infection of non-phagocytic cells is an active penetration.

Cytochalasin B is a fungal metabolite known to have many biological effects, among them inhibition of phagocytosis and of cytoplasmic cleavage (Carter, 1967). The purpose of the present investigation was to examine the effect of cytochalasin B on the interaction of 3 different species of *Leishmania* with mouse peritoneal exudate cells in vitro.

Materials and methods

Organisms

The origin and past histories of *Leishmania tropica major, L. donovani* and *L. enriettii* used in the present investigation is given elsewhere (Rezai et al., 1975). *Leishmania* promastigotes were grown in modified NNN medium (Lemma and Schiller, 1964). For experimental purposes the organisms were harvested in their logarithmic phase of growth (Ardehali, 1974).

Macrophage culture

Outbred mice were injected i.p. on two consecutive days with 1 ml of Hanks' Balanced Salt Solution (HBSS). On the third day the animals were killed by dislocation of the neck and each inoculated i.p. with 3.0 ml of Eagle's Basal Essential Medium (EBEM) containing 5 U of heparin per ml (Choay Laboratories, Paris, France) and 10% (v/v) fetal bovine serum (Flow Laboratories, Maryland, USA). The peritoneal exudate cells collected from several mice were pooled and samples counted in an improved Neubauer haemocytometer. The viability of the cells was determined by Trypan blue dye exclusion test and the cell density of the suspension was adjusted to 2×10^6/ml. 1.5 ml of cell suspension was added to Leighton tubes containing 10.5×50 mm coverslips. The peritoneal cell cultures were incubated at 37° C in an incubator with 5% CO_2. After 2 h of incubation the non-adherent cells were washed away and fresh medium was added to the tubes containing adherent cells.

The prepared cells were challenged with *Leishmania* promastigotes 24 h after incubation at 37° C in the presence of 5% CO_2.

Preparation of cytochalasin B solution

Cytochalasin B was obtained commercially (Ralph N. Emanuel Ltd., Wembley, Middlessex) and was dissolved in dimethyl sulfoxide (DMSO). Final dilutions were made in EBEM.

Leishmania-macrophage interaction

Leishmania promastigotes were suspended in EBEM and added to glass adherent cells at a ratio of 8–10/1. The promastigotes were allowed to remain at 37° C for 2 h. The infected macrophages were washed thoroughly to remove the non-phagocytized organisms and fresh medium was added. At various time intervals the macrophages were fixed with absolute methanol for 1 min and stained with Giemsa. A total of 100 macrophages were observed and the number of infected cells was recorded.

Effect of cytochalasin B on interaction of macrophages and parasites

To test for the effect of cytochalasin B, 1.5 ml of promastigotes (2×10^7/ml) were added to p.e.c. cultures in the presence of the drug at concentrations of 2.5, 5.0 or 10 μg/ml. After 2 h at 37° C, the cultures were washed to remove non-phagocytosed organisms and cytochalasin B; fresh medium was then added, and incubation was resumed. After 6 h, coverslips were processed for microscopical observation as described above.

In some experiments, macrophages were pretreated for 2 h with the drug prior to addition of parasites in drug-containing medium. To test for reversibility of the effect of cytochalasin B, macrophages were drug-treated for 2 h, then washed and reincubated for 20 min in drug-free medium prior to addition of drug-free parasites for a 2-h period. Cultures were then washed and reincubated for 6 h prior to harvest as indicated above.

Results

Infection of mouse peritoneal exudate cells with Leishmania promastigotes

Direct observations of *Leishmania*-macrophage interaction by inverted microscope showed that, within a few minutes after promastigotes were added to the p.e.c., the promastigotes began to attach to the main body or pseudopods of the cells by the anterior end of their flagella. Contact seemed to be at random, with the attachment of several organisms to a single cell. After 8 h of incubation, the cells were examined, and a total of 100 p.e.c. were counted. The rates of infection (mean $\pm$ SD) of the macrophages were respectively, 53.5 $\pm$ 5% for *L. enriettii,* 52.3 $\pm$ 5% for *L. donovani* and 11.7 $\pm$ 2% for *L. tropica.* The number of amastigotes within the exudate cells varied from 1–6 with an average of 4.

The immediate effect of cytochalasin B on Leishmania-macrophage interaction

From dose-response studies, it was observed that 2.5, 5.0 and 10.0 μg/ml of cytochalasin B had no toxic effect for mouse p.e.c. or *Leishmania* promastigotes as measured by the Trypan blue dye exclusion test.

When the above concentrations of the drug and the promastigotes were added to the monolayers at the same time, it was noticed that cytochalasin B at a concentration of 10 μg/ml had exerted some negative effect on the uptake of *L. donovani.* The infection rate of the treated macrophages was 39.0 $\pm$ 6.0% in contrast to 49.0 $\pm$ 4.0% in the control cultures. However, the percentage of

Table 1. Effect of cytochalasin B (CB) on phagocytosis of *Leishmania* by mouse macrophages

Organism	2 h pulse of macrophages with CB prior to addition of parasites	Percent infection of macrophages at various concentrations of CB (μg/ml)			Controls (DMSO)***
		10	5	2.5	0
L. donovani	no	39 ± 6*	51 ± 3	49 ± 4	49 ± 4
	yes	1.0**	< 1.0	0	52.3
L. enriettii	no	47 ± 6	49 ± 1	46 ± 4	52 ± 3
	yes	0	< 1.0	< 1.0	53.5
L. tropica	no	11 ± 3	9 ± 2	11 ± 3	11 ± 1
	yes	1.0	< 1.0	< 1.0	11.7

* Mean ± S.D. of 3 replicates
** Mean of 3 replicates
*** Controls treated with dimethyl sulphoxide only

infected cells in presence of 5.0 and 2.5 μg/ml of the drug was similar to the control values (Table 1). With respect to *L. enriettii* and *L. tropica*, the uptake of promastigotes by the peritoneal cells was very much similar to control values at all concentrations of cytochalasin B.

Effect of pretreatment of macrophages with cytochalasin B on the uptake of Leishmania

In this experiment peritoneal exudate cells were treated with cytochalasin B for a 2-h period and then infected with *Leishmania* promastigotes. As a control, untreated cell cultures were inoculated with the promastigotes, and the mode of attachment as well as the percentage of infection was evaluated. It was observed that although the *Leishmania* became attached to the surface of the cells in both control and test cultures, uptake of all three species by the treated peritoneal cells was considerably inhibited. All three concentrations tested inhibited the uptake of promastigotes by the p.e.c. to the same degree. In the test cultures only 1–2% of the p.e.c. became infected after 6 h, whereas nearly 50% of the control cells became infected with *L. donovani* and *L. enriettii* and nearly 12% with *L. tropica*. The results are presented in Table 1.

Reversibility of cytochalasin B-induced phagocytic inhibition

24-h old cultures of p.e.c. were treated with cytochalasin B at various concentrations for a 2-h period. After treatment, the drug was removed and the cells were inoculated with fresh medium for 20 min and then inoculated with *Leishmania* promastigotes. The results are presented in Table 2.

Pretreatment of the monolayers with cytochalasin B at a concentration of 10 μg/ml completely inhibited the uptake of all *Leishmania* organisms, even

Table 2. Reversibility of cytochalasin B-induced phagocytic inhibition

Organism	Percent infection of macrophages after removal of cytochalasin B, following preincubation with various concentrations of the drug (μg/ml)			Control
	10	5	2.5	0
L. donovani	0	8.5*	15.5	53.5
L. enriettii	0	6.5	8	52.5
L. tropica	0	1	1.5	11.7

* Each number represents mean values based on results obtained from two replicates.

after removal of the drug. Washed macrophages pretreated with 5or 2.5 μg/ml of cytochalasin B, however, did recover some of their phagocytic activity toward *L. donovani* and *L. enriettii* but not towards *L. tropica.* The infection with *L. donovani* in presence of 2.5 μg/ml of cytochalasin B was almost twice that of *L. enriettii.* With *L. tropica* the infection rate was almost similar to the pretreated macrophages, i.e. around 1%.

Discussion

Experiments on *Leishmania*-macrophage interaction show that when p.e.c. of mice were infected with various species of *Leishmania* promastigotes, the percent infection was higher with *L. donovani* and *L. enriettii* than with *L. tropica.* Since *Leishmania* organisms show some degree of host specificity (Garnham, 1971), one might expect this specificity to be reflected in the phagocytic avidity of the peritoneal exudate cells of these animals toward the different *Leishmania* species. Our findings, however, demonstrated that although our outbred strain of mice becomes easily infected with *L. tropica,* the p.e.c. from the same mice phagocytosed *L. tropica* with the least efficiency. 6 h after infection, only 11% of the p.e.c. became infected with *L. tropica* whereas approximately 50% became infected with *L. donovani* and *L. enriettii.* It appears that either the in vitro phagocytic ability does not reflect the avidity of the macrophages in vivo, the ability to phagocytose the *Leishmania* parasite is not a critical factor in the outcome of infection, or the failure to rapidly phagocytose the parasites somehow leads to an active infection. The differences in the uptake of *Leishmania* by mouse p.e.c. can not be attributed solely to the physiological state and membrane differences of macrophages in vivo and in vitro but also to the differences between induced peritoneal macrophages and skin macrophages which are normally infected by *L. tropica.*

It can be postulated that what determines the establishment of an infection is not so much the phagocytic activity of the macrophages but rapid transforma-

tion of promastigote to amastigote form, resistance to lysosomal enzymes or prevention of access of lysosomal enzymes to phagocytic vesicles.

When p.e.c. of mice were treated for a 2-h period with cytochalasin B and then infected with *Leishmania* promastigotes, 1–2% of the cells were infected in contrast to the controls which had infection rates of close to 50% for *L. donovani* and *L. enriettii* and 10% for *L. tropica.* The rate of infection of these treated cells did not vary for the three species of *Leishmania* tested. These results confirm the work of Alexander (1975) who used mouse p.e.c., similar concentrations of the drug and *Leishmania mexicana.* On the other hand, when epimastigotes of *Trypanosoma cruzi* were used as the test organism he noticed that cytochalasin B treatment did not affect the infection rate of the p.e.c. The fact that *T. cruzi* not only infects the cells of the reticuloendothelial system in a mammalian host but also infects the cells which are not usually phagocytic, such as myocardial or neuroglia cells, may suggest active penetration of *T. cruzi* into non-phagocytic cells.

Although the mechanism underlying the action of cytochalasin B on cells is not clear, the antiphagocytic activity of this drug is well established in mouse p.e.c. (Klaus, 1973). Allison et al. (1971) demonstrated that cytochalasin B used at low concentrations disrupts microfilaments of macrophages and as the result inhibits phagocytosis. These investigators have suggested that cell motility and infolding of the membrane, both of which are necessary for phagocytosis, comes from contraction of microfilaments.

Studies on *Leishmania*-macrophage interaction have led to several interpretations. The *Leishmania* amastigotes are not motile, and it has been assumed that these are phagocytosed by the cell. However, in the case of *Leishmania* promastigotes which are actively motile, controversial reports exist. Dvorak and Schmunis (1972), using mouse p.e.c. and promastigotes of *Leishmania donovani,* concluded that infection of macrophages is by engulfment of the organisms by the cell and not by active penetration of the organisms. On the other hand, because *Leishmania mexicana* can infect non-phagocytic cells, such as conchal cartilage of the ear and pigment cells in the epithelium, it has been suggested by Lainson and Strangeways-Dixon (1963) that the infection of these cells by *Leishmania* is by active penetration.

It has been noted that *Leishmania* promastigotes are unable in vitro to gain access to the cells of low phagocytic ability, namely baby hamster kidney cells, human amnion cells and rhesus monkey cells. Infection of dog sarcoma cells by *Leishmania* promastigotes has been demonstrated (Akiyama and McQuillan, 1972; Lewis, 1974). It is well known that these types of cells are, in fact, migratory histiocytes.

From the present investigation, it is concluded that in vitro infection of mouse p.e.c. by the three different species of *Leishmania* promastigotes used, is an active phagocytosis by the cells rather than active penetration of the organisms. It is also of interest to note that although mice of our outbred strain get

easily infected with *L. tropica*, the peritoneal exudate cells of these animals had the lowest infection rate in comparison with *L. enriettii* and *L. donovani.*

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