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Species-specific Interaction of *Leishmania* Promastigotes with Macrophages: Effect of Previous Incubation of the Host Cells with Enzymes, Monosaccharides and Lectin

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ABSTRACT

Leishmania spp., the agents of human visceral, post kala-azar dermal and cutaneous leishmaniasis, are the intracellular parasites that must be recognised and internalised by host macrophages for its pathogenesis. The involvement of specific ligand-receptor interaction necessary for the entry of different Leishmania promastigotes into murine macrophages were investigated. The effect of two enzymes on the attachment and uptake of Leishmania promastigotes by macrophages which might be found in the wound caused by the bite of the phlebotomine sandfly vector were also examined. With the two sugars tested, mannose and galactose were produced a significant inhibition of uptake in viscerotropic and dermotropic form of leishmaniasis, respectively. Mild trypsinization inhibited the uptake potential in all cases, with much higher value in the case of post kala-azar dermal leishmaniasis. Treatment of neuraminidase to macrophages enhanced attachment in all three strains to some extent. These results indicate that the nearly similar type of inhibition profile of dermal and cutaneous strains were probably due to the similar type of skin habitat.

INTRODUCTION

The surface of the parasites is vital in their interactions with the host to bring about successful parasitism. The establishment of intracellular parasitisms in any host-parasite system involves a series of cellular events. Parasite-macrophage interaction is a common phenomenon of many protozoa, viz. few species of Plasmodium, Eimeria tenella, Toxoplasma gondii, Besnoitia jellisoni, Leishmania spp., Trypanosoma cruzi, Nosema michaelis, Pneumocystis spp. etc. (Bray 1982). In leishmaniases, a disease complex resulting from infection by protozoa of the genus Leishmania (family Trypanosomatidae), attachment to the macrophage membrane is a prerequisite to phagocytosis. The subsequent intracellular survival, differentiation and multiplication of Leishmania species culminate in all the clinical manifestation of the infection. Promastigote-macrophage binding is a membrane phenomenon akin to both

immunological and non-immunological ligand-receptor interactions. Such ligand--receptor site binding is also common among bacteria (Lis and Sharon 1986). Possible ligands on the parasite surface include a 60-65 KD glycoprotein (gp 63, the promastigote surface protease) (Bouvier et al. 1985, 1987; Etges et al. 1985; Russell and Wilhelm 1986; Ouaissi 1988), a complex lipophosphoglycan (Turco et al. 1984; Handman and Goding 1985; Handman 1990; Englund 1993; Descoteaux and Turco 1993), other fucose and mannose containing glycoconjugates (Palatnik et al. 1989, 1990) and the third component of complement (Russell 1987; Puentes et al. 1988). Macrophage receptors involved in parasite capture may include the complement receptor 3; through fixation to iC3B, to the Arg-Gly-Asp sequence of gp63 or to externally exposed sugars (Russell and Wilhelm 1986; Blackwell et al. 1985; Wilson and Pearson 1986; Wozenkraft et al. 1986; Wozenkraft and Blackwell 1987; Ouaissi 1988; Russell and Wright 1988), the fibronectin receptor (Rizvi et al. 1988), receptors that recognize carbohydrates (Blackwell et al. 1985; Wilson and Pearson 1986) and a receptor for advanced glycosylation end products (Mosser et al. 1987). The enhancement of macrophage infectivity of L. major by salivary gland material of sandfly is perhaps also a receptor-mediated event (Theodos and Titus 1993).

The objective of this work is to establish the involvement of specific ligand--receptor interaction presumably necessary for the entry of Leishmania promastigotes into murine macrophages in vitro. We have also studied the effect of two enzymes on the attachment and uptake of Leishmania promastigotes by macrophages which might be found in the wound caused by the bite of the phlebotomine sandfly vector. The modes of attachment and uptake between promastigotes and macrophages in species-specific manner were also evaluated.

MATERIALS AND METHODS

Leishmania donovani of visceral leishmaniasis (VL), L. donovani of post kala-azar dermal leishmaniasis (PKDL) and L. tropica of cutaneous leishmaniasis (CL) were used in this study. The detail of the strains and growth conditions were previously described (Ghosh et al. 1990).

Macrophage collection and culture were followed after Handman and Spira (1977). The peritoneal cavity of BALB/c mice (CDRI strain) stimulated 3 d previously with 2 ml of 4% thioglycollate broth (Difco, USA). After killing by cervical dislocation exudate cells of the mice were harvested from peritoneal cavity by syringe with RPMI-1640 tissue culture medium (Gibco labs, USA) containing 200 U/ml penicillin, 200 µg/ml streptomycin and 5 U/ml heparin. The isolated cells were pooled, centrifuged and resuspended in culture medium. One ml of the cell suspension containing 10^6 cells was plated on flying sterile coverslip (20 mm^2) in plastic tissue culture dish (Steriware, India) in groups of ten for each strain. The cells were allowed to adhere for 2 h at 37°C in a humid atmosphere of 5% CO_2, 95% air (Forma Scientific, Inc. Ohio, USA). After incubation the culture medium was removed from each plate and washed twice with assay medium. The assay medium was the same except 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo, USA) which replaced the fetal calf serum (RPMI-BSA) (Datta and Majumder 1986).

Stationary phase promastigotes (Bandyopadhyay et al. 1991) were collected by centrifugation at 1,650 x g for 10 min, counted in haemocytometer, suspended in assay medium (RPMI-BSA) and adjusted to the desired concentration, i.e. 80 : 1 (promastigote : macrophage). After incubating each set of dish for 60 min under the same conditions as stated above, the coverslips with infected macrophages were dried, fixed in methanol and stained with giemsa. Site, number of attached promastigotes and the average number of amastigotes per

macrophage were determined by examining at least 100 macrophages per coverslip at 1,000 x by Zeiss standard microscope. All experiments were done in triplicate. The average values and the standard deviations were determined.

Trypsinization of cells was carried out with crystallised enzyme (EC 3.4.21.4; Merck AG, Darmstadt, FRG) for 30 min at 500 µg/ml (Bray 1983). Macrophages were also treated with 0.1 U/ml neuraminidase (EC 3.2.1.18) from Clostridium perfringens (Sigma) in PBS, pH 7.4 for 30 min also. Peritoneal macrophage preparations (10^6 cells) were incubated for 30 min at 100 µg/ml of wheat germ agglutinin (WGA, Sigma) in assay medium. Galactose and mannose (Merck AG) at 100 mM in assay medium were employed in this experiment. After 30 min incubation samples were processed for binding estimation.

The percentage of infected macrophages, the mean number of intracellular parasites per infected macrophages and the endocytic index were calculated by the method of Araujo-Jorge and DeSouza (1984, 1986). The endocytic index was calculated by multiplying the percentage of infected macrophages and the mean number of parasites per infected cell. In each experiment the endocytic indexes were normalized by taking the value obtained for the control as zero. Since some treatment induced a decrease in the endocytic indexes in relation to the control, the results are expressed as the positive or negative percent variation at the endocytic index. Statistical analysis was performed using the F-test of the endocytic indexes obtained. The results are express as mean ± standard deviations. Differences higher than 20% of control were considered to be significant.

RESULTS AND DISCUSSION

The rate of interiorization of promastigotes to macrophages was different in different strains of Leishmania. The basic parameter of the promastigote--macrophage interaction of control experiment was shown in Table 1. Results showed that the percentage of infected macrophages was from 16.3 ± 1.52 to 34.3 ± 8.14 (Table 1). The L. donovani of PKDL strain showed highest ability to attach and penetrate the macrophages. However, the VL and CL had very close type of infective ability. But the endocytic index was higher in PKDL (59.9 ± 0.90) and much lower in CL (22.8 ± 3.00) and VL (20.0 ± 1.90). It is presume that as the PKDL is the terminal form of VL in certain cases, it has got genetic pre-adaptation for establishment in the mononuclear phagocytes in a better way than that of the VL form. On the otherhand, there is evidence that the attraction is not exerted upon the promastigotes, but instead the macrophage seeks the promastigotes by the complement product C5a from promastigotes which is chemotaxis for macrophages (Bray 1983).

Attachment of motile parasite to the macrophages took place predominantly by the flagellar tip. Few promastigotes were rarely seen to have attached by the cell body. Simultaneous flagellar and cell body attachment were also seen. On the contrary of our average result, few promastigtes were shown to penetrate into macrophage through their posterior end without any participation of flagellum. The mode of attachment in different strains of Leishmania was almost of similar nature. Interactions between Leishmania promastigotes and macrophage membranes display the characteristics of a receptor-ligand interaction, such as specificity, saturability and competitive inhibition (Mauel 1990). The consensus theory regarding Leishmania-macrophage interaction is the promastigotes approach the macrophage and do so in most cases flagellum

Table 1. Basic parameters on the Leishmania-macrophage interaction for 60 min in control experiment*

Parameter	Leishmaniasis		
	Visceral	Post kala-azar dermal	Cutaneous
Percentage of infected macrophages	16.3 ± 2.08	34.3 ± 8.14	16.3 ± 1.52
Mean number of parasites per infected macrophages	1.2 ± 0.11	1.8 ± 0.52	1.4 ± 0.17
Endocytic index	20.0 ± 1.96	59.9 ± 0.90	22.8 ± 3.00
Parasites/100 macrophages	20.0 ± 2.00	59.0 ± 1.00	22.6 ± 2.51

* Data from three representative experiments.

first (Zenian et al. 1979). Moreover, this may be by any part of the promastigotes (Chang 1979). On the contrary, Akiyama and Haight (1971) proposed that the parasites entered the host cells with posterior end first. Our result also support the theory of attachment through any part of the body but the flagellar tip is the favoured point of attachment (Ghosh 1989). The binding ligands must be present on both the flagellar and the cell-body section of promastigotes (Ghosh et al. 1990) because they can attach to macrophages via either side. No marked differences are observed in different strains of Leishmania regarding their mode of attachment. Interestingly, L. donovani of PKDL strain showed the highest ability to attach and penetrate the macrophage.

Treatment of macrophages with neuraminidase slightly improved the invasion of macrophages in all 3 strains (Fig. 1). But these slightly improved endocytic indexes were not considered significant. The level were 4.4%, 4.6% and 10.9% in VL, PKDL and CL cases, respectively. The PKDL strain showed comparatively higher invasive index than the other two. It was seen that proteolytic enzyme, i.e. trypsin when applied to the macrophages reduced the attachment as well as uptake significantly. The highest inhibition was seen in PKDL (42.3%) strains (Fig. 1B). However, border line inhibition was observed in VL (12%) and CL (18.8%) strain (Fig. 1A-B). It can be said from the results reported here that the macrophage receptors are sensitive to proteolytic enzyme, i.e. trypsin. Similar type of result was also observed in Trypanosoma cruzi (Alcantara and Brener 1980) and Corynebacterium parvum (Ogmundsdottir et al. 1978). Chang (1981) demonstrated that mild trypsinization of macrophages caused an approximately 75% reduction in their capacity of binding promastigotes of L. donovani. In PKDL, trypsin drastically reduced the percentage of endocytic index relative to the control, i.e. 42.3%. Twelve and 18.8% inhibition are also observed in VL and CL cases. These two values are considered as nearly significant. During host-parasite interaction, macrophages produce complement factors including properdin and promastigote cleave compfement, partly at least by the alternative complement pathway and bind C3bi (Bray 1983). C3bi and mannose on promastigotes surface could then bind

to complement type 3 receptor and mannose-fucose receptor. These receptors are trypsin sensitive and consequently reduced the uptake ratio. It can be concluded from our results that both dermotropic form of Leishmania have encountered more trypsin-sensitive complement receptors of macrophages during their interaction in vitro. Treatment of neuraminidase to macrophages assist attachment in all three strains of Leishmania. However, the values are not statistically significant, nevertheless, neuraminidase slightly increases the ratio of uptake probably by reducing the negative charges on the macrophage (Ogmundsdottir et al. 1978; Bray 1983).

Galactose showed a strong inhibition of about 32% in the case of PKDL strain (Fig. 1B). A nearly significant level of inhibition (18%) was seen in the case of CL strain with the same sugar (Fig. 1C), whereas it was almost similar as control in the VL strain (Fig. 1A). The uptake of L. donovani promastigotes of VL was moderately inhibited (12%) by addition of mannose (Fig. 1A). However, it has no effect on the uptake of promastigotes of the PKDL and CL strain (Fig. 1B-C). Incubation of macrophages with WGA did not markedly altered the endocytic indexes. In VL strain, WGA can slightly reduce the relative percentage of endocytic index (2.5%). But in PKDL and CL strains, it acts in opposite way. In the otherwords, 1.3% and 0.7% elevation of relative endocytic index were found in the above two dermotropic strains (Fig. 1A-C). With the two sugars tested, the statistical analysis of the results clearly show that in the absence of FCS, mannose and galactose produce a significant inhibition of uptake in viscerotropic and dermotropic form of leishmaniasis, respectively. I employed only these two sugars on the basis of their significant presence on the surface of viscerotropic and dermotropic Leishmania promastigote (Ghosh et al. 1990).
An interesting point to observe is that the inhibitory effect of uptake the promastigotes of visceralizing strain by mannose is highest (12%) whereas in PKDL and CL strain it is 1.5% and 3%, respectively. On the otherhand, galactose induced strong inhibition in dermotropic form (32.5% and 18.5%) than viscerotropic form (2.9%). This result clearly correlates with our lectin binding test result which demonstrates that mannose and galactose are predominantly present on the surface of viscerotropic and dermotropic form of Leishmania, respectively. Our results, when compared with others (Chang 1981; Datta and Majumder 1986; Hernandez et al. 1986; Chakraborty and Das 1988) reveal some interesting features. Our observations are not in well agreement with that of Hernandez et al. (1986) and Datta and Majumder (1986). They suggest that β-D-galactose, glucose, N-acetylgalactosamine showed binding inhibition in L. donovani promastigotes, whereas sialic acid, fucose and arabinose did not inhibit the binding (Hernandez et al. 1986). On the contrary, Chang (1981) demonstrated that D-galactose and N-acetyl-D-galactosamine to be non-inhibitory in L. donovani. We only observed significant inhibition by galactose in dermotropic form of leishmaniasis, whereas mannose showed strong inhibition in viscerotropic form only. Chakraborty and Das (1988) suggested that internalization of L. donovani promastigotes by hamster macrophage is predominantly due to exposed sugars on the surface of the parasites and occurs primarily via the mannose/N-acetylglucosamine receptor. Our result partly supports this mannose-mediated attachment in L. donovani of VL strain.

Very recently Guy and Belosevic (1993) have demonstrated the types of receptors are used by amastigotes of L. major to gain entry into macrophages. They postulated that promastigotes and amastigotes of L. major both share and have distinct receptors for entry into macrophages. The observed differences on the receptors used may reflect the altered environments to which

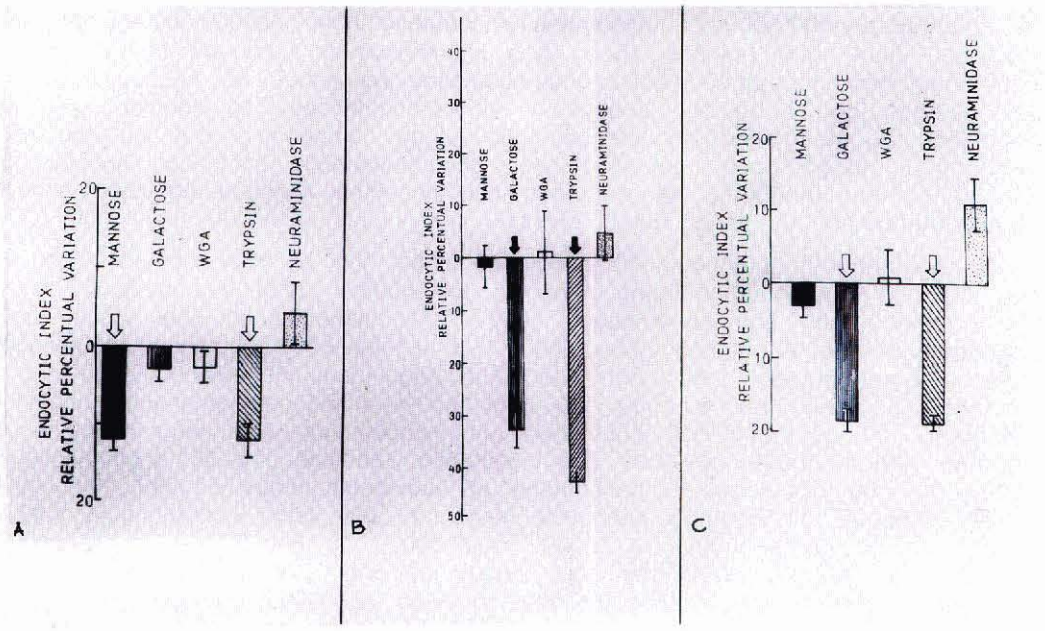

Fig. 1. The effect of incubation of murine macrophages in the presence of mannose, galactose, wheat germ agglutinin, trypsin and neuraminidase on the binding in vitro of Leishmania donovani of visceral leishmaniasis **(A)**, L. donovani of post kala-azar dermal leishmaniasis **(B)** and L. tropica of cutaneous leishmaniasis **(C)** to the macrophage cells. Solid arrows indicate those situations in which the effect observed is statistically significant and hollow arrows in which the effect observed borderline significant as compared to control. Histograms above and below the control zero (0) line, signify the stimulation and inhibition effect, respectively. The values shown are the mean of three experiments and bars indicate the standard deviation.

the promastigotes and amastigotes are exposed. This may have caused differential selection pressures for alternate mechanisms of entry of the two stages of Leishmania species into macrophages. In this study, nearly similar type of inhibition profile of PKDL and CL strains were probably due to the similar skin habitat (Bhattacharya et al. 1993). In other words, the differences between the macrophage-binding ligands on the different Leishmania species, as well as the inhibition profile may also play a role in determining parasite tropism (Russell and Talamas-Rohana 1989).

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