Effect of bioamines on uptake of promastigotes of *Leishmania donovani* by hamster peritoneal macrophages

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Summary. Epinephrine and norepinephrine inhibit attachment of *Leishmania donovani* promastigotes to cultured hamster peritoneal macrophages. The inhibition was significant at catecholamine concentrations of 10^{-4} and 10^{-5} M and occurred when they were added to the cell mixtures, or after pre-treatment of either macrophages or parasites. Inhibition of attachment after pre-treatment was less marked than when the catecholamines were added to parasite-cell mixtures. Similar results were obtained with dibutyl cyclic AMP, cholera toxin, theophylline, and cadaverine which raise intracellular cyclic AMP (cAMP). Pre-treatment of parasites or macrophages with the bioamines elevated the intracellular cAMP concentration. It is suggested that the inhibitory effect on the host-parasite interaction is mediated through cAMP.

Introduction

Macrophages are the host cells of *Leishmania donovani*, the aetiological agent of visceral leishmaniasis. The interaction between macrophages and the parasite involves recognition, binding and internalisation. Attachment of the parasite to the host cell is thought to be mediated by some form of receptor-ligand interaction¹ and may involve Fc or C3b receptors or a lectin-like glycoprotein receptor on the macrophage cell surface.^{2,3}

Although macrophage-parasite association is of prime importance for the initiation of infection, little is known about its regulation. Cyclic nucleotides and neurohormones are known to modulate several physiological and immunological reactions in phagocytic cells. Phagocytosis is retarded in the presence of catecholamines or cyclic AMP (cAMP). Whether this effect is due to internal or external concentrations of cAMP acting on the cell surface is uncertain.^{4,5} Macrophage phagocytosis of starch or opsonised sheep erythrocytes is inhibited by cAMP and agents that raise cAMP concentrations. We have evaluated the involvement of catecholamines in the initial interaction between promastigotes of *L. donovani* and macrophages.

Materials and methods

Chemicals

Epinephrine (bitartrate crystals), norepinephrine (bitartrate salt), N⁶-2'-O-dibutyl adenosine 3':5'

cyclic monophosphate (dibutyl cAMP), theophylline, cholera toxin, cadaverine, HEPES buffer, L-glutamine and Giemsa stain were purchased from Sigma Chemical Co., St Louis, MO, USA. Medium RPMI 1640, fetal bovine serum (FBS), streptomycin and penicillin were purchased from Gibco, Grand Island, NY, USA. Sodium thioglycollate was purchased from Difco Laboratories, Detroit, MI, USA. cAMP assay kits were purchased from Amersham International plc, Amersham, Bucks.

Macrophage culture

The complete medium for macrophage culture was RPMI 1640 supplemented with FBS (heat-inactivated at 56°C for 30 min) 10%, 25 mM HEPES buffer, 4 mM L-glutamine, streptomycin 100 µg/ml and penicillin 100 U/ml. Macrophages were harvested from the peritoneal cavity of Golden Syrian hamsters which had been stimulated 3 days earlier by intraperitoneal (i.p.) injection of thioglycollate broth 4% w/v. On the day of an experiment, each animal was given 20 ml of serum-free RPMI medium by i.p. injection. After gentle abdominal massage, peritoneal fluid was collected, pooled and centrifuged aseptically. The pellet was resuspended in complete RPMI medium. Cells were counted in a haemocytometer. Monolayers on glass coverslips (22 mm²) were prepared from 0.2-ml samples (1×10^6 cells/ml) in 35-mm sterile petri dishes.⁶ They were incubated in CO₂ 5%-air 95% at 37°C for 2 h to allow adhesion. Non-adherent cells were removed by washing with supplemented RPMI 1640 without FBS, 2 ml of fresh complete culture medium was added to each petri dish, and the macrophages were incubated overnight at 37°C.^{7,8}

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L. donovani promastigotes

These were of strain UR6 and were routinely cultured in modified Ray's medium as described elsewhere.⁹ Promastigotes were suspended in complete RPMI medium, washed twice and counted in a haemocytometer after suitable dilution.

Assay

A promastigote-macrophage association assay was developed to measure the effect of the test drugs on parasite adherence to the host cell. The assays were done under three experimental conditions to assess the site of action of the drugs.

In test A, the effect of drugs and chemicals on the ability of macrophages to take up untreated parasites was assessed. The monolayer was pre-treated with the test agent in 1 ml of medium at appropriate concentrations for 1 h at 37°C and then washed in agent-free complete RPMI 1640. Parasite suspension (1 ml) was added to the monolayer in the promastigotes: macrophage ratio 5:1 and the mixture was incubated for 1 h at 37°C. Non-adherent parasites were removed by rinsing twice in the dish with serum-free RPMI 1640 medium. The monolayer was then fixed with methanol and treated with Giemsa stain.

Test B measured the effect of drugs on promastigote adherence to untreated macrophages. The test differed in that the parasite suspensions were pre-treated with the test agent by incubating for 1 h at 26°C. Samples were taken after 1 h to check parasite motility. After 1 h, the parasite suspensions were washed twice by centrifugation at 900 rpm for 5 min before they were resuspended in complete RPMI 1640 medium and added to the untreated monolayers. After incubation at 37°C for 1 h, the monolayers were rinsed, fixed and stained as before.

Test C measured the effects of the presence of agents on parasite-macrophage interaction. It differed in that the agents were added together with the parasites to the macrophage monolayer without any pre-treatment and incubated for 1 h at 37°C before rinsing and staining.

Each experiment was done at least three times with matching agent-free controls. Coverslips were examined by microscopy ($\times 400$). Counts of at least 100 macrophages were done in each case to determine the number with attached parasites and the number of parasites bound per cell. Data were analysed statistically by Students' *t* test.

Cyclic AMP assay

Macrophage and parasite preparations used to measure cAMP levels were prepared as follows. Adherent macrophages (as described above) were removed from sterile coverslips with a "rubber policeman" and suspended in RPMI 1640 medium at a concentration of 10^7 cells/ml. Parasites were

similarly suspended in RPMI 1640 medium at 10^7 cells/ml. Both macrophage and parasite cell suspension were centrifuged for 15 min at 3000 rpm. Cell pellets were resuspended in 2 ml of PBS, pH 7.4, and an equal volume of trichloroacetic acid (TCA) 10% was added to each tube and mixed thoroughly. The mixtures were centrifuged after 15 min and the supernate of each tube was extracted four times with water-saturated ether to remove TCA. The water layer was lyophilised and reconstituted with 130 μ l of sodium acetate buffer, pH 6.2; 50- μ l volumes were used to measure cAMP with a commercial radioimmunoassay kit (Amersham International) according to the manufacturer's instructions. Similar procedures were performed with treated macrophages and parasites. Results were expressed as the picomolar concentration of cAMP in relation to a reference curve obtained with solutions of cAMP of known concentration.

Results

Attachment of *L. donovani* to hamster peritoneal macrophage monolayers was inhibited by epinephrine and norepinephrine after pre-treatment of either the macrophages or the parasites with the catecholamines (table I, A and B). Adhesion of *L. donovani* to hamster peritoneal macrophages was also inhibited by both agents when these were incubated with the parasite-macrophage preparation (table I, C). At concentrations of 10^{-4} M and 10^{-5} M, the catecholamines significantly reduced the percentage of macrophages with attached parasites. The reduction seen at 10^{-6} M was not significant (table I).

The effect of these catecholamines on the number of parasites bound per macrophage are shown in table II. By this criterion it was noted that pre-treatment of parasites (test B) inhibited attachment less than did tests A and C. Pre-treatment of the parasite did not cause significant change in motility at 1 h.

Agents known to raise cellular cAMP, i.e., cholera toxin, theophylline and cadaverine, were then tested. All these agents inhibited parasite-macrophage binding. Pre-treatment of macrophages with these agents produced greater inhibition than was found with pre-treatment of parasites. All the agents significantly reduced both the proportion of macrophages with attached parasite and the number of parasites bound per cell (table III).

Similarly, direct addition of dibutyryl cAMP significantly reduced the percentage of infected macrophages (table IV) and the number of parasites attached per macrophage (table V).

A significant rise in intracellular cAMP level was observed when the parasites were pre-treated with adrenaline and noradrenaline. The stimulation was greater with adrenaline than with noradrenaline. An increase of the intracellular cAMP level was also noted when macrophages were pre-treated with these bioam-

Table I. Effect of catecholamines on *L. donovani* attachment with hamster peritoneal macrophages

Experimental condition	Percentage of macrophages attached with <i>L. donovani</i> (mean and SEM)						
	Control	Epinephrine (M)			Norepinephrine (M)		
		10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
Test A. Pre-treated macrophages	63.1 SEM 2.5	44.7 SEM 3.3† (29.2%)*	51.0 SEM 3.6 (19.1%)	56.7 SEM 4.3 (10.2%)	46.7 SEM 3.5† (26.0%)	50.0 SEM 2.1† (20.7%)	56.7 SEM 4.3 (10.2%)
Test B. Pre-treated parasites	52.7 SEM 2.12	37.0 SEM 2.1† (29.7%)	41.7 SEM 2.7§ (20.9%)	48.3 SEM 3.2 (8.3%)	40.0 SEM 2.4† (24.1%)	41.0 SEM 2.3§ (22.2%)	48.7 SEM 2.6 (11.5%)
Test C. Macrophages and parasites treated together	62.1 SEM 2.63	44.3 SEM 2.9† (28.6%)	52.7 SEM 3.3 (15.2%)	54.3 SEM 4.3 (12.5%)	37.0 SEM 3.5† (40.4%)	51.3 SEM 4.7 (17.3%)	54.0 SEM 3.4 (13.0%)

* Figures in parenthesis are results expressed as percentage inhibition.

† p < 0.01.

‡ p < 0.02.

§ p < 0.05.

Table II. Effect of catecholamines on macrophage-*L. donovani* interaction

Agent	Molar concentration	Number of <i>L. donovani</i> attached per macrophage (mean and SEM)		
		Test A Pre-treated macrophages	Test B Pre-treated parasites	Test C Parasites and macrophages treated together
Epinephrine	0	3.28 SEM 0.54 (0)*	2.50 SEM 0.24 (0)	3.28 SEM 0.54 (0)
	10 ⁻⁴	1.53 SEM 0.01† (53%)	1.61 SEM 0.08† (36%)	1.64 SEM 0.19† (50%)
	0	3.04 SEM 0.43 (0)	2.50 SEM 0.24 (0)	3.04 SEM 0.43 (0)
	10 ⁻⁵	1.84 SEM 0.20 (39%)	1.71 SEM 0.14† (32%)	1.70 SEM 0.08† (44%)
	10 ⁻⁶	2.20 SEM 0.40 (28%)	2.19 SEM 0.16 (12%)	2.00 SEM 0.26 (34%)
Norepinephrine	0	3.28 SEM 0.54 (0)	2.50 SEM 0.24 (0)	3.28 SEM 0.54 (0)
	10 ⁻⁴	1.55 SEM 0.05† (53%)	1.58 SEM 0.07† (37%)	1.67 SEM 0.10† (49%)
	0	3.04 SEM 0.43 (0)	2.50 SEM 0.24 (0)	3.04 SEM 0.43 (0)
	10 ⁻⁵	1.74 SEM 0.19† (43%)	1.76 SEM 0.06† (30%)	1.74 SEM 0.06† (43%)
	10 ⁻⁶	2.12 SEM 0.35 (30%)	2.17 SEM 0.18 (13%)	1.94 SEM 0.10 (36%)

* Figures in parenthesis are results expressed as percentage inhibition.

† p < 0.05.

ines. The stimulatory effect of these bioamines on cAMP formation was higher at 10⁻⁴ M than that at 10⁻⁵ M. The extent of stimulation by adrenaline and noradrenaline was almost the same (table VI).

Discussion

This study reveals that catecholamines, added cAMP and agents that elevate intracellular cAMP, inhibit the attachment of *L. donovani* to cultured macrophages. Epinephrine, norepinephrine, dibutyl cAMP, theophylline, cadaverine and cholera toxin, each significantly reduced the attachment of parasites to macrophages, regardless of their mode of application. Inhibition took place in the presence of the agents in the incubation mixture and also after pre-

treatment of either cell type. Thus, both ability of the macrophage to take up the parasite and of the parasite to adhere to macrophages were sensitive to the agents.

Leishmania-macrophage interaction is critical for the initiation of visceral leishmaniasis. This is thought to involve specific recognition and interaction of lectin-like receptors on the macrophage with sugar ligands on the parasite surface.^{10,11} Attachment is followed by a complex series of events. It is possible to recognise internalisation or phagocytosis by the macrophage, followed by transformation of the parasite, leading to its proliferation.³ Macrophage interaction with leishmania,¹² and trypanosomes⁵ is sensitive to cAMP and agents that induce it. These agents have also been shown to be important in models of phagocytosis^{4,13} but the site and nature of this modulation is not understood.

Table III. Effect of cholera toxin, theophylline and cadaverine on macrophage-*L. donovani* interaction

Experimental conditions	Agents	Percentage of macrophages with attached <i>L. donovani</i> (mean and SEM)	Average number of <i>L. donovani</i> attached per macrophage (mean and SEM)
Test A. Pre-treated macrophages	None	76.4 SEM 3.83	2.89 SEM 0.19
	Cholera toxin		
	50 ng/ml	40.0 SEM 3.23† (47.7%)	0.80 SEM 0.05†
	25 ng/ml	55.2 SEM 3.22‡ (27.8%)	1.10 SEM 0.14†
	Theophylline		
	10 ⁻⁴ M	54.4 SEM 2.58‡ (28.8%)	1.14 SEM 0.12†
	10 ⁻⁵ M	55.2 SEM 2.86 (27.8%)	1.11 SEM 0.08†
Test B. Pre-treated parasites	Cadaverine		
	10 ⁻⁴ M	59.6 SEM 3.77§ (22.0%)	1.32 SEM 0.14†
	10 ⁻⁵ M	62.0 SEM 3.18† (18.9%)	1.56 SEM 0.15†
	None	62.4 SEM 1.94	1.76 SEM 0.11
	Cholera toxin		
	50 ng/ml	54.8 SEM 2.33¶ (12.2%)	1.32 SEM 0.09§
	25 ng/ml	57.6 SEM 3.02 (7.7%)	1.40 SEM 0.14¶
	Theophylline		
	10 ⁻⁴ M	49.6 SEM 2.74‡ (20.6%)	1.15 SEM 0.07†
	10 ⁻⁵ M	55.2 SEM 3.13 (11.6%)	1.16 SEM 0.07‡
	Cadaverine		
	10 ⁻⁴ M	48.8 SEM 2.25‡ (21.8%)	1.28 SEM 0.12‡
	10 ⁻⁵ M	54.4 SEM 2.61 (12.9%)	1.35 SEM 0.10‡

* Figures in parenthesis are results expressed as percentage inhibition.

† p < 0.001.

‡ p < 0.01.

§ p < 0.02.

¶ p < 0.05.

Table IV. Effect of cAMP on attachment of *L. donovani* with hamster peritoneal macrophages

Experimental condition	Percentage of macrophages with attached <i>L. donovani</i> (mean and SEM)			
	Control	10 ⁻⁴ M	10 ⁻⁵ M	10 ⁻⁶ M
Test A. Pre-treated macrophages	63.11 SEM 2.5	44.3 SEM 3.2† (29.8%)*	50.0 SEM 2.8‡ (20.7%)	56.33 SEM 3.3 (10.7%)
Test B. Pre-treated parasites	80.0 SEM 4.33	56.40 SEM 3.86‡ (42%)	68.0 SEM 3.06 (51%)	75.20 SEM 5.44 (54%)
Test C. Macrophages and parasites treated together	62.1 SEM 2.63	46.66 SEM 3.5§ (24.9%)	47.33 SEM 2.8§ (23.8%)	55.66 SEM 5.6 (10.4%)

* Figures in parenthesis are results expressed as percentage inhibition.

† p < 0.01.

‡ p < 0.02.

§ p < 0.05.

Table V. Effect of cAMP on macrophage-*L. donovani* interaction

Molar concentration of cAMP	Number of <i>L. donovani</i> attached per macrophage (mean and SEM)		
	Test A Pre-treated macrophage	Test B Pre-treated parasites	Test C Parasites and macrophages treated together
0	3.28 SEM 0.54 (0)*	2.50 SEM 0.24 (0)	3.28 SEM 0.54 (0)
10 ⁻⁴	1.47 SEM 0.23† (55%)	1.58 SEM 0.10† (37%)	1.52 SEM 0.09† (54%)
0	3.04 SEM 0.43 (0)	2.50 SEM 0.24 (0)	3.04 SEM 0.43 (0)
10 ⁻⁵	1.75 SEM 0.24 (42%)	1.73 SEM 0.08† (31%)	1.73 SEM 0.10† (43%)
10 ⁻⁶	2.00 SEM 0.32 (34%)	2.05 SEM 0.10 (18%)	1.90 SEM 0.28 (37%)

* Figures in parenthesis are results expressed as percentage inhibition.

† p < 0.05.

Table VI. Effect of catecholamines on intracellular concentrations of cAMP of parasites and macrophages

Agent present	Concentration (M)	pM cAMP/10 ⁷ cell (mean and SEM)	
		<i>L. donovani</i>	Macrophage
Control	...	0.63 SEM 0.05	2.77 SEM 0.42
Adrenaline	10 ⁻⁴	6.5 SEM 0.58*	10.9 SEM 0.91†
	10 ⁻⁵	2.17 SEM 0.17*	3.2 SEM 0.43
Noradrenaline	10 ⁻⁴	3.2 SEM 0.41†	9.1 SEM 0.8†
	10 ⁻⁵	1.0 SEM 0.09‡	2.5 SEM 0.34

*p < 0.001.

†p < 0.01.

‡p < 0.02.

cAMP inhibition of peritoneal macrophagocytosis was demonstrated with opsonised sheep erythrocytes¹⁴ and *Candida albicans*¹⁵ and the latter authors also described inhibition of polymorphonuclear leukocyte phagocytosis. Interestingly, cAMP at lower concentrations reportedly stimulates uptake of aggregated bovine serum albumin by macrophages.¹⁶ In the *Trypanosoma cruzi*-macrophage model, cAMP has been found to inhibit initial attachment.⁵

Phosphodiesterase inhibitors such as theophylline increase cAMP levels in leishmanias.¹² They also inhibit macrophagocytosis of *T. cruzi*⁵ and the inhibition was associated with a rise in cell cAMP.⁵ As in the present study, neither motility nor viability of the parasite was affected by the agents.⁵

Therefore, there seems ample evidence that cAMP is a modulator of host-parasite interactions in several

species and with several phagocytic cells. A cAMP-dependent protein kinase has not so far been demonstrated in leishmanias. However, our study shows that leishmania promastigotes, as well as the macrophages, are sensitive to cAMP manipulation in such a way as to alter their ability to attach when they subsequently interact. Elevation of intracellular cAMP concentration has also been noted in the presence of bioamines, which suggests that the inhibitory effect on host-parasite interaction is mediated through cAMP. However, the exact mechanism through which cAMP modulates the interaction is not known. Nevertheless, our evidence suggests that cAMP acts at the key steps of cell attachment by modulating surface receptors in both host and parasite in *L. donovani* infection.

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