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The effect of parasitization by *Leishmania mexicana mexicana* on macrophage function in vitro[3]

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Summary

Macrophages infected with amastigotes of *Leishmania mexicana mexicana* as compared to normal macrophages show decreased migration both randomly and through a 5 μm pore in response to a known chemotaxin, an increased ability to pinocytose and an increased bactericidal ability. Unless very heavily parasitized their ability to phagocytose is unaltered. Parasitized macrophages are unaltered in their ability to secrete extracellularly lysosomal enzymes, prostaglandins and lysozyme in response to known stimuli, or to kill target cells in an antibody dependent cell mediated cytotoxicity assay.

Key words: macrophages; *Leishmania mexicana;* phagocytosis; migration; bactericidal activity; enzyme secretion; prostaglandins; antibody dependent cell cytotoxicity.

Introduction

Direct information concerning the effect of intracellular protozoan parasites upon macrophage function biochemically or physically is scanty. Handman et al. (1979) produced evidence for a reduced H-2^d antigen expression on the surface of BALB/c mice macrophages infected with *Leishmania tropica*, and a murine macrophage line J 774G8 when infected in vitro with *L. mexicana amazonensis* displayed an increased ability to take up fluorescein-tagged dextran (Chang, 1980).

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Simple in vitro techniques are easily available for the examination of migration, pinocytosis, phagocytosis, antibody dependent cell mediated cytotoxicity, bactericidal activity and enzyme and prostaglandin secretion by macrophages. In this paper the effect of parasitization by *L. mexicana mexicana* on some of these macrophage functions in vitro is recorded.

Materials and Methods

The parasites used were amastigotes of *L. mexicana mexicana* (LV4) maintained in the rump skin of TO outbred mice. The macrophages studied were obtained from the peritoneal cavities of TO outbred mice of 2–3 m of age, with or without prior intra-peritoneal stimulation by 2 ml of 100 μg Concanavalin A (Con A) in tissue culture medium 199 (199) 2 days previously or of 2% soluble starch in physiological saline 3 days previously or were from the femurs of TO mice. Cells were washed from the peritoneal cavities in physiological saline containing 50 iu/ml heparin. Bone marrow cells were flushed from femurs in 199.

The cells were washed two times in Hanks' balanced salt solution (HBSS) then allowed to settle in 5% heated new born calf serum (HNBCS) in 199 on glass coverslips in Leighton tubes or onto treated plastic in Nunc 25 cm^2 flasks (Gibco, Paisley) or directly in 96 well cluster plates (Costar, Cambridge, Mass., USA) for antibody dependent cell mediated cytotoxicity (ADCC) assays. Peritoneal cavities were infected by inoculation with 10^8 amastigotes or *L. mexicana mexicana* and the cells removed as above 10 min later to obtain unattached infected cells for macrophage migration studies. Cells which had attached to glass or plastic were washed twice with 199 at 36° C after 1 h in the case of starch stimulated cells, 4 h in the case of Con A stimulated cells, 6 h in the case of bone marrow cells and 24 h in the case of unstimulated peritoneal cells and then covered with 10% HNBCS/199 in the case of peritoneal cells and 10% heated foetal calf serum (HFCS) in 199 in the case of bone marrow cells.

Migration

Infected and non-infected starch stimulated peritoneal cells were washed and sedimented in 50 λ capillary tubes. The tubes were snapped off at the cell/supernatant surface and the tube containing about 3 mm of cells anchored in small wells containing 0.5 ml of 10% HNBCS/199 then left at 36° C for 24 h when the area of migration of the cells from the capillary tubes was measured. Infected and non-infected Con A stimulated or unelicited peritoneal cells were placed in Boyden type chambers and the chemotaxic effect of 0.6 mg casein/ml in 199 measured as described previously (Bray, in press).

Pinocytosis and phagocytosis

Cells from starch and Con A stimulated and non-stimulated peritoneal cavities and from bone marrows which had been allowed to settle in 10% HNBCS/199 or 10% HFCS/199 in Leighton tubes or tissue culture flasks were allowed to rest for 24 h at 36° C and half were then infected with amastigotes of *L. mexicana mexicana*, approximately 5 per cell. Bone marrow cells were allowed to grow for 6 days in 10% HFCS/199 and then infected as before. 24 h after infection infected and uninfected cultures were fed at 36° C with either 5/cell of glutaraldehyde fixed sheep red blood cells (G-SRBC), sheep red cells treated with rabbit haemolysin (H-SRBC) or sheep red blood cells (SRBC) or with 10% HFCS/199 containing 0.5% of an emulsion of Oil Red O, paraffin oil and lipopolysaccharide (LPS) (prepared by adding 10 mg of LPS and 50 mg of Oil Red O to 1 ml of HBSS and 0.5 ml of light paraffin oil and sonicating the mixture at 0° C for 15 min at 70 watts). After 30 min the cells fed with the Oil Red O preparation were washed 4 times in PBS then shaken with 1 ml of p-dioxan for 24 h. The preparation was spun and the Red Oil O content of the dioxan in each culture was measured in a spectrophotometer at 525 nm. Ninety minutes after feeding with G-SRBC or 30 min after feeding SRBC and H-SRBC the cells on the cover slips from Leighton tubes were fixed

in aqueous Bouin's fluid for 15 min, washed in 70% alcohol then water, stained in 10% Giemsa at ph 7.2 for 1 h, differentiated in 50% water 50% acetone, dehydrated in acetone, cleared in xylene followed by euparal essence and mounted in green euparal. The percentage of cells infected, the number of amastigotes/100 cells, the percentage of cells containing SRBC, G-SRBC and H-SRBC and the number of SRBC, G-SRBC or H-SRBC/100 cells was determined in the infected cultures and the percentage of cells containing SRBC, G-SRBC or H-SRBC and the number of SRBC, G-SRBC or H-SRBC/100 cells was determined in the uninfected cultures.

Secretion

Macrophages were allowed to settle in 10% HNBCS/199 in the case of peritoneal cells or in 10% HFCS in the case of bone marrow cells in flasks for 24 h (6 days for bone marrow cells) then amastigotes were added and left for 24 h. Other cells were kept uninfected as controls. Con A (50 μg/ml) SRBC, (5 per cell) H-SRBC (5 per cell), LPS (20 μg/ml) or zymosan (50 particles/cell) were added for six hours or 24 h. After each step or each change of medium the supernatant was kept and tested using Sigma (Poole, Dorset) kits for the content of lactate dehydrogenase, alkaline phosphatase, acid phosphatase, B-glucuronidase and by radioimmunoassay for prostaglandin (PG)-like activity measured as PG E_2, 6-keto PG $F_{1\alpha}$ (stable metabolite of prostacyclin) and thromboxane B_2 (Granström and Kindahl, 1978). Lysozyme content in supernatants was measured by lysis of *Micrococcus lysodeikticus* (Gordon et al., 1974).

Antibody dependent cell mediated cytotoxicity

Starch stimulated macrophages from CBA mice were allowed to adhere for 1 h at 37° C in 96 well clusters as 4×10^4 cells per well in 100 μl of 10% HFCS/RPMI 1640 tissue culture medium (RPMI) then washed twice in HBSS at 37° C. Varying concentrations of or no amastigotes of *L. mexicana mexicana* and *L. major* were added in 30% HFCS/RPMI and left for 24 h at 37° C then washed in HBSS and the medium replaced with 100 μl fresh medium (30% HFCS/RPMI) containing varying concentrations of target cells and mouse anti-target cell serum and left for 4 h at 37° C. The target cells were chicken red blood cells (CRBC) or BW 5147 thymoma cells which had previously been labelled by incubation for 1 h at 37° C in 10% HFCS in phosphate buffered saline containing 100–200 μCi ^{51}Cr as $Na_2\ ^{51}CrO_4$ (Amersham International Ltd., Amersham). All experiments were done in triplicate.

After 4 h incubation all plates were centrifuged and the supernatant removed. 100 μl of 1N NaOH was added to the remaining cells and these removed. All supernatants and cell homogenates were then counted over 20 sec for γ emissions in a Mini-Assay gamma counter (Mini-Instruments Ltd., Boreham on Crouch, Essex). The percent ^{51}Cr released =

$$\frac{2 \times A - C}{A + B - C} \times 100\%$$

where A = count for supernatant, B = count for cells and C = count for supernatants from cell targets in the absence of macrophages (background). The percent ADCC was defined as percent ^{51}Cr released in the presence of anti-serum less that released in its absence.

Microbicidal activity

The ability of infected and uninfected macrophages to kill *Staphylococcus aureus* was measured by the method of Bar-Eli and Gallily (1979). Briefly a suspension of *S. aureus* was added to infected and uninfected starch elicited peritoneal macrophages in 10% HNBCS/199 in Leighton tubes as 100 bacteria/cell and incubated for 30 min at 36° C. Free bacteria were then removed by repeated washing and the macrophages incubated for a further hour. Cells were then lysed in 0.1% Triton $\times$100 and suitable dilutions of the lysate plated out on *S. aureus* medium and counted after 24 h. Some cultures were examined by phase microscopy to determine if the uptake of *S. aureus* was similar in infected and uninfected macrophages.

Table 1. Migration of infected and uninfected macrophages in vitro

Experiment	Area of random migration at 24 h	
	uninfected	infected
1	82.4 (7.5)	44.6 (6.7)[2]
2	56.3 (8.6)	35.4 (1.1)[1]
3	68.4 (5.3)	51.1 (8.2)[1]
4	96.3 (5.6)	70.0 (10.1)[1]
5	62.4 (6.0)	42.9 (5.0)[1]

The cell migration was projected onto graph paper at a magnification of 20×. The magnified area of migration was measured. The results are in cm^2.

Experiment		Chemotaxis	
		No. of macrophages penetrating filter	
		uninfected	infected
1	at 1 parasite/cell	47.1 (6.3)	36.2 (3.2)[2]
2	at 5 parasites/cell	37.0 (6.8)	24.9 (3.8)[3]
3	at 10 parasites/cell	52.7 (5.9)	20.4 (3.5)[3]

Results of Student t test: [1] $p<0.05$; [2] $p<0.005$; [3] $p<0.001$. – Standard errors in parenthesis

Results

Migration

The migration of infected macrophages was inhibited significantly compared to uninfected macrophages. In 5 different sets of triplicate experiments infected cell migration into medium was inhibited to 54%, 63%, 75%, 72% and 69% of uninfected cell migration (Table 1). In 3 different sets of triplicate experiments infected cell migration through filters in a gradient of a known chemotaxin (casein) was inhibited to (in ascending order of parasitization) 77%, 67% and 39% of uninfected cell migration (Table 1).

Pinocytosis and phagocytosis

Pinocytosis of LPS/paraffin oil (see Table 2) as measured by endotoxin/paraffin oil/Oil Red O uptake was greater in infected cells than uninfected cells regardless of the source of the cells.

Phagocytosis was unaffected by parasitization provided the number of amastigotes per cell was below 10 (see Table 3). When the infection of cells was very heavy e.g. 5823 amastigotes/100 cells no SRBC were taken up. At infections of between 2000 and 3000 amastigotes per 100 peritoneal cells phagocyto-

Table 2. Pinocytosis. Uptake of Oil Red O/paraffin/LPS into macrophages in vitro measured as content of Oil Red O per 10^6 macrophages expressed as absorption in a spectrophotometer at 525 mm

Type of cell	Infected	Uninfected
Starch stimulated	38.0 (5.2)	21.1 (4.1)[1]
Unelicited	23.2 (4.4)	14.8 (2.3)[1]
Bone marrow	7.7 (1.7)	3.5 (0.8)[1]

[1] Student t test: $p<0.05$. – Standard errors in parenthesis

sis of sheep red cells was inhibited. Phagocytosis of large numbers of fresh SRBC will kill macrophages, and this happened with uninfected bone marrow macrophages but the presence of large numbers of amastigotes protected macrophages by limiting the number of SRBC taken up.

Secretion

There were no differences in secretion by infected cells as compared to uninfected cells of lactate dehydrogenase, alkaline and acid phosphatases, β-glucuronidase, prostaglandins E_2 and $F_{1\alpha}$, thromboxane and lysozyme in response to stimulation by Con A, LPS, zymosan, SRBC and H-SRBC. Perhaps more important the act of phagocytosis of amastigotes failed to increase the secretion into the medium of any of the enzymes or prostaglandins measured.

Antibody dependent cell mediated cytotoxicity

Both uninfected and infected macrophages showed only low levels of lytic activity towards CRBC and BW5147 thymoma cells in the absence of specific antiserum. In general, with either of 2 concentrations of antiserum, against either target and over varied infection levels (0.2 to 20 amastigotes per cell), no significant difference was found in % ADCC at any given effector/target cell ratio between uninfected and infected macrophages. Table 4 gives representative results of these experiments. Similar results were obtained using macrophages infected with *L. major*.

Microbicidal activity

The uptake of *S. aureus* was similar in cultures of infected and uninfected macrophages. Infection improved the bactericidal ability of macrophages. The ratio of viable *S. aureus* counts in infected over uninfected cells at various dilutions were 88/150, 61/92, 28/46 (Table 5). The differences were significant in the first two dilutions.

Table 3. Phagocytosis. Number of sheep red blood cells per 100 macrophages in in vitro cultures 24 h after five sheep red blood cells per macrophage had been fed to the macrophage cultures

Type of macrophage	No. of SRBC[1]		No. of GSRBC[1]		No. of HSRBC[1]		No. of SRBC[1]	
	infected below 10 am*/cell	uninfected	infected below 10 am*/cell	uninfected	infected below 10 am*/cell	uninfected	infected above 10 am*/cell	uninfected
Starch stimulated .	42 (12.3)	21 (8.2)	278 (23.4)	343 (38.9)	920 (123.1)	1030 (111.5)	70 (5.2)[3]	183 (11.9)[3]
Unelicited	58 (8.7)	57 (9.3)	125 (15.8)	136 (10.3)	334 (41.0)	345 (38.3)	372 (21.2)[3]	765 (20.3)[3]
Bone marrow	27 (4.2)	26 (3.9)	52 (8.4)	55 (7.9)	82 (6.7)	96 (11.0)	522 (62.8)	–[2]

[1] SRBC = Sheep red blood cells; GSRBC = Glutaraldehyde fixed sheep red blood cells; HSRBC = Haemolysin treated sheep red blood cells

[2] Above 10 SRBC/cell all cells destroyed

[3] Student t test: $p<0.01$

*am = amastigotes of *Leishmania*

Table 4. ADCC activity of uninfected and *Leishmania*-infected peritoneal macrophages against chicken red blood cells (CRBC) and BW 5147 thymoma cell targets

Macrophage	E:T ratio[1]	Percent antibody dependent cell mediated cytotoxicity			
		CRBC		BW 5147	
		Specific antiserum concentration			
		10^{-5}	10^{-3}	10^{-5}	10^{3}
Uninfected	0.1	1.7 (0.2)	1.4 (0.3)	4.9 (0.5)	2.2 (0.1)
	1.0	18.0 (1.0)	21.0 (0.5)	3.7 (1.5)	4.4 (0.3)
	5.0	32.0 (2.0)	21.5 (2.0)	12.2 (0.3)	11.5 (0.5)
	10.0	22.0 (1.0)	17.5 (1.0)	30.0 (1.0)	17.0 (1.0)
	25.0	23.7 (1.1)	16.3 (1.6)	49.0 (3.0)	20.1 (1.2)
L. m. mexicana-[2] infected	0.1	3.5 (0.5)	3.0 (0.2)	2.1 (1.0)	3.0 (0.5)
	1.0	16.7 (0.5)	17.4 (1.6)	3.6 (0.2)	2.4 (0.5)
	5.0	30.0 (1.2)	18.0 (1.3)	14.0 (1.5)	8.2 (1.5)
	10.0	31.0 (1.5)	14.5 (1.1)	31.0 (1.5)	16.0 (0.7)
	25.0	23.5 (0.6)	16.0 (1.5)	49.0 (2.0)	25.0 (1.0)

[1] Effector cell to target cell ratio
[2] approx. 5 amastigotes per macrophage infection level

Standard deviation in parenthesis. Differences greater than 4% are statistically significant. Student t test: $p<0.05$.

Table 5. Macrophage killing of *Staphylococcus aureus*. 100 bacteria per macrophage fed to in vitro cultures of macrophages. After 90 min cells are lysed and bacteria at 3 different dilutions grown in nutrient medium for 24 h followed by counting

Dilution of bacteria before growth	Counts of *S. aureus* from:	
	infected cells	uninfected cells
1/20	92.5 (5.45)	150.3 (4.24)[1]
1/80	60.1 (6.32)	91.8 (5.35)[1]
1/320	28.0 (12.1)	46.2 (6.58)[2]

[1] Student t test: $p<0.001$
[2] Not significant
Standard errors in parenthesis

Discussion

The presence of various intracellular bacteria and latex is known to inhibit the random migration of human blood leucocytes in vitro (Allgower and Block, 1949; Martin and Chaudhuri, 1952; Bryant et al., 1966) but these results applied to neutrophils rather than monocytes. As for macrophages Jones and

Byrne (1980) have stated "... in neither case (infection with *Toxoplasma* or chlamydia) does the host cell (macrophage) appear to be significantly impaired by the presence of the replicating organisms".

Work reported here shows that infection of macrophages with *Leishmania* in vitro impairs both random motility and movement through a 5 μm pore in response to chemotaxic gradient. The presence of 10 or so leishmanial mastigotes inside the macrophage will impart a certain rigidity to the cell and this could explain the inhibition of its ability to pass through a 5 μm pore or indeed through the vascular endothelium and thus its ability to reach a site of inflammation. The reason why parasitization with leishmanial amastigotes should impair random migration is less easy to explain. Obviously cytoplasmic flow might be restricted, endocytosis of parasites might deplete the surface membrane of some critical elements of the plasma membrane, rigidity might impair the deformity and plasticity necessary for migration and all of these could affect movement of the cell.

Chang (1980) demonstrated an increased endocytosis of fluorescein labelled dextran by *L. m. amazonensis*-infected macrophages in vitro. This is confirmed by the report here that light and moderate infection with amastigotes of *Leishmania* increases pinocytosis as measured by the uptake of LPS in oil. Ingestion of heat killed bacteria is known to increase the ability of neutrophils to phagocytose further particles (Cohn and Morse, 1960) but increased ability of actively parasitized macrophages to pinocytose has only been reported very recently (Chang, 1980). In contrast macrophages with light or medium loads of parasites do not show increased phagocytosis of sheep red cells whether untreated, glutaraldehyde-fixed or treated with haemolysin. Very heavily infected macrophages do show an impaired ability to phagocytose sheep red cells. This contrasts with the results of Rabinovitch and Gray (1968) who showed that uptake of killed bacteria increased the ability of macrophages subsequently to ingest fixed red cells.

Secretion by macrophages in vitro of lysosomal enzymes, prostaglandins and lysozyme in response to LPS, zymosan, SRBC and Con A stimulation was not significantly altered by parasitization. Unstimulated peritoneal cells produced much greater amounts of PGE_2 than starch elicited or bone marrow macrophages as has been noted before but were unaffected in this regard by parasitization. It is well known that the stimulants used here normally produce a burst of secretion of lysosomal enzymes, prostaglandins and lysozyme (Gordon et al., 1974; Bonney et al., 1978; Davies et al., 1974; Schorlemmer et al., 1977; Schnyder and Baggiolini, 1978; Weidemann et al., 1976) and while in the work reported here, this increased production of macrophage secretions followed stimulus for all cell types it did not occur to a greater or lesser degree in *Leishmania*-infected macrophages. *Leishmania*-infected macrophages were in no way impaired or enhanced in their ability to act as effector cells in antibody dependent cell mediated cytotoxicity assays.

Bactericidal activity of macrophages in vitro was increased by parasitization by *L. m. mexicana;* the ability to kill *S. aureus* being increased by a factor of about 1.6 for starch elicited peritoneal macrophages. This is in contrast to the reported deficiency of the microbicidal activity of macrophages loaded with SRBC (Gill et al., 1966).

On the whole macrophages are little disturbed by the phagocytosis and subsequent presence of leishmaniae in their phagosomes in vitro and this would agree well with the lack of oxidative metabolic burst following phagocytosis of *Toxoplasma* (Wilson et al., 1980).

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