In vitro stimulation of metacyclogenesis in *Leishmania braziliensis*, *L. donovani*, *L. major* and *L. mexicana*

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SUMMARY

Promastigotes of *Leishmania braziliensis*, *L. donovani*, *L. major* and *L. mexicana* recently derived from tissue amastigotes were cultured in Schneider's *Drosophila* medium supplemented with 20 % (v/v) heat-inactivated foetal calf serum and 25 μg gentamicin sulfate/ml at pH 5·5. These cultures produced more metacyclic promastigotes in their stationary-phase populations than others cultured at pH 7·0. Metacyclic promastigotes possessed a short ($\leqslant 8$ μm) and narrow ($\leqslant 1·5$ μm) cell body with a flagellum twice or more the length of the cell body. Promastigotes from acidic cultures were more resistant to complement-mediated lysis and more infective *in vivo* than those grown at neutral pH. These results demonstrate that induction of metacyclogenesis by acidic pH is a response conserved across a variety of species of *Leishmania*.

Key words: *Leishmania* spp., promastigote, metacyclic, culture, infectivity, differentiation.

INTRODUCTION

Parasites of the genus *Leishmania* adopt 2 main morphological forms during their life-cycle; the amastigote, which divides within cells of the vertebrate host, and various types of promastigote, which are found in the gut lumen of the sandfly.

Early investigators believed that there was 1 particular form of promastigote in the sandfly which was adapted for life in the vertebrate host, now known as the metacyclic promastigote (Shortt, Smith & Swaminath, 1931; Adler & Ber, 1941). More recently, morphological and biochemical differences between dividing midgut forms and those found anteriorly in sandflies have been recognized, and many workers have investigated metacyclic forms and metacyclogenesis (see for example, Killick-Kendrick, 1990; Bates, 1994).

In a previous study, metacyclogenesis in *L. mexicana* was induced by culturing promastigotes at acidic pH (Bates & Tetley, 1993). The metacyclic promastigotes produced were resistant to complement-mediated lysis and were highly infective to macrophages *in vitro*. In this present study, we have extended this investigation of metacyclogenesis to include species causing different types of clinical disease: *L. braziliensis*, *L. donovani*, *L. major* and *L. mexicana*. Metacyclic promastigotes and multiplicative promastigotes of these 4 species were characterized in terms of morphology, complement resistance and infectivity *in vivo*.

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MATERIALS AND METHODS

Parasites

Infections of *L. donovani* (MHOM/ET/67/HU3; LV9) and *L. braziliensis* (MHOM/BR/84/LTB300) were maintained in hamsters. *L. mexicana* (MNYC/BZ/62/M379) and *L. major* clone FV-1 (MHOM/IL/80/Friedlin) infections were maintained in BALB/c mice. Promastigotes were obtained by transformation of tissue amastigotes at 26 °C in HO–MEM (Berens, Brun & Krassner, 1976) supplemented with 10 % (v/v) heat-inactivated foetal calf serum (FCS) and 25 μg gentamicin sulfate/ml, followed by culture for 2–4 days. Recently transformed promastigotes were subcultured into Schneider's *Drosophila* medium (Life Technologies) supplemented with 20 % (v/v) heat-inactivated FCS and 25 μg gentamicin sulfate/ml at 26 °C. The initial pH of this medium was adjusted either to 7·0 or 5·5 using 1 M HCl or 1 M NaOH as required and 10 ml volumes were cultured in 25 cm² tissue culture flasks using air as the gas phase. Cell counting was performed using an Improved Neubauer haemocytometer under phase-contrast microscopy.

Light microscopy

Thin smears were prepared from promastigotes, air dried, fixed in absolute methanol, and stained for 10 min in 10 % (v/v) Giemsa's stain in 10 mM sodium phosphate buffer (pH 7·2). Slides were examined at ×1000 magnification under oil immersion. Measurements were made using an Olympus microscope fitted with a drawing tube. A ruler and a test slide

were used to calibrate the microscope so that each 2 μm on the test slide was equal to 1 mm on the ruler. Cell body length (L) and width (W) were measured for a minimum of 100 promastigotes in each slide examined.

Complement-mediated lysis

Promastigotes in the exponential phase or stationary phase (first day where there was no increase in cell density) of growth *in vitro* were harvested by centrifugation and washed in Hanks' balanced salt solution (HBSS) twice, then resuspended to a density of 10^7 cells/ml in HO–MEM supplemented with 10% (v/v) heat-inactivated FCS, pH 7·0. Doubling dilutions of guinea-pig serum from 1:1 to 1:128 were prepared in the same medium in 96-well flat-bottom plates, in a volume of 100 μl/well. Promastigote suspensions were added (100 μl), the plate was covered with Parafilm and incubated at 26 °C for 1 h. At the end of the incubation period, 40 μl of 50 mM EDTA were added (final concentration 8·3 mM), to stop complement-mediated lysis. Examination was performed by pipetting 5 μl volumes on a glass slide and covering with a 22 × 22 mm cover-slip. In this way, promastigotes were trapped between the slide and the cover-slip and movements of viable promastigotes were easily observed. Viability was estimated for each serum dilution by examining 100 promastigotes and determining the percentage with moving flagella. The concentration of serum that lysed 50% of promastigotes was used to compare relative resistance of different cultures to complement-mediated lysis.

Infectivity *in vivo*

Stationary-phase promastigotes cultured at pH 7·0 and 5·5 were washed twice in HBSS and brought to a density of 1×10^8 cells/ml for *L. donovani*, 2×10^7 cells/ml for *L. mexicana* and *L. major* and 4×10^8 cells/ml for *L. braziliensis*. Two groups of 12 female BALB/c mice were used for each species, one was inoculated with promastigotes cultured at pH 7·0 and the other with promastigotes cultured at pH 5·5.

Mice to be infected with *L. donovani* were inoculated i.v. in the tail vein with 0·2 ml containing 2×10^7 promastigotes and killed 2 weeks after inoculation. Their livers were removed and impression smears made after weighing. Smears were fixed in absolute methanol for 1 min, stained in 10% (v/v) Giemsa/phosphate buffer for 25–30 min and examined under the microscope. A magnification of × 400 was used to find an area on the slide with a uniform layer of hepatocyte nuclei. Amastigotes were counted /1000 liver cell nuclei at × 1000 magnification. The mean number of amastigotes/liver cell nucleus was multiplied by the weight of the liver (mg) to obtain the LDU (Leishman–Donovan

units) index of infectivity (Bradley & Kirkley, 1977). A *t*-test of significance was used to compare statistically the LDU values using the Minitab program.

Mice to be inoculated with *L. mexicana*, *L. major* and *L. braziliensis* were shaved at the base of the tail and inoculated subcutaneously at this site with 0·05 ml containing 10^6 promastigotes for *L. mexicana* and *L. major* and 2×10^7 promastigotes for *L. braziliensis*. Length and width of lesions were measured weekly with calipers for 10 weeks and the lesion size determined.

RESULTS

Light microscopy

Thin smears were made from exponential- and stationary-phase promastigotes cultured at pH 7·0 or 5·5 and examined. The value of $L \times W$ was calculated for each promastigote and any having a value of $\leqslant$ 12 was considered a culture-derived metacyclic promastigote. Such metacyclic promastigotes possessed short ($\leqslant 8$ μm), narrow ($\leqslant 1·5$ μm) cell bodies and long flagella twice or more the length of the cell body. Metacyclic promastigotes first appeared in acidic cultures during the exponential phase and reached prevalences of 77% for *L. donovani*, 78% for *L. mexicana*, 75% for *L. major* and 49% for *L. braziliensis* by the stationary phase (Fig. 1). The percentages of metacyclic promastigotes in equivalent stationary-phase cultures initiated at pH 7·0 were 11% for *L. donovani*, 29% for *L. mexicana*, 10% for *L. major* and 12% for *L. braziliensis*.

Complement resistance

Exponential- and stationary-phase promastigotes grown at acidic and neutral pH were subjected to complement-mediated lysis. The percentage of viable promastigotes was estimated using movement of the flagellum as a sign of viability. Stationary-phase promastigotes cultured at acidic pH showed a higher resistance to complement-mediated lysis than those grown in neutral pH (Table 1) in all 4 of the species tested, although each showed different relative resistance to complement-mediated lysis. To enable comparison between species, the concentration of serum required to cause 50% lysis in acidic and neutral cultures was expressed as a ratio, indicating 4-fold to 16-fold increases in resistance.

Infectivity *in vivo*

Stationary-phase promastigotes of *L. donovani* cultured at pH 7·0 and 5·5 were used to infect 2 groups of 12 female BALB/c mice. Mice were killed 2 weeks after inoculation, their livers removed and the LDU determined for each. Out of 12 mice inoculated with promastigotes cultured at pH 5·5, 11 had amastigotes
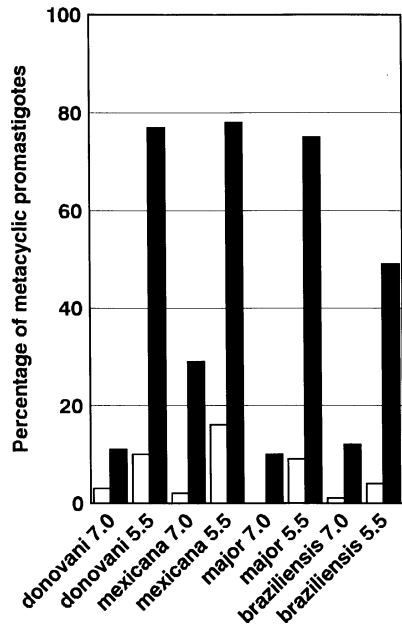
Fig. 1. Percentage of metacyclic promastigotes in exponential phase (□) and stationary phase (■) for cultures of *Leishmania donovani*, *L. mexicana*, *L. major* and *L. braziliensis* at pH 7·0 and 5·5.

Table 1. Relationship between culture pH and complement resistance of stationary-phase promastigotes

Species	pH of culture	Percentage serum causing 50 % cell lysis	Increase in resistance
Leishmania donovani	7·2	6·25	4-fold
	5·5	25	
Leishmania mexicana	7·2	1·56	8-fold
	5·5	12·5	
Leishmania major	7·2	3·125	16-fold
	5·5	50	
Leishmania braziliensis	7·2	0·78	8-fold
	5·5	3·125	

of *L. donovani* in their livers and the average LDU in this group was 276 ($\pm$ 54 s.e.). On the other hand, only 3 out of 12 mice inoculated with promastigotes cultured at pH 7·0 had amastigote infections with an average LDU of 14·1 ($\pm$ 7·4 s.e.) in this group. The LDU values were compared using a *t*-test, which indicated a significant statistical difference between the 2 values ($P = 0.0007$). This clearly indicated that promastigotes cultured at acidic pH were more infective than those cultured at neutral pH. In other words, mice infected with promastigotes cultured in acidic pH had approximately 5.5×10^7 amastigotes/
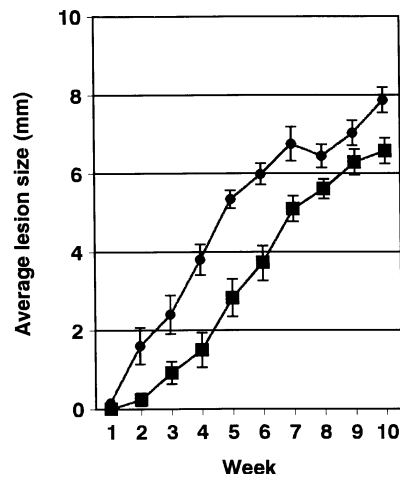

Fig. 2. Development of *Leishmania mexicana* lesions in BALB/c mice infected with stationary-phase promastigotes cultured at pH 7·0 (■——■) and 5·5 (●——●). Each group contained 12 animals. The bars represent 1 standard error of the mean.
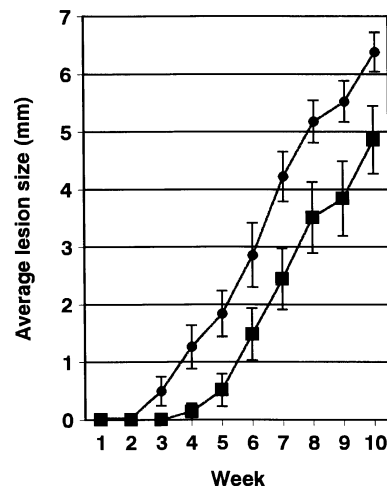

Fig. 3. Development of *Leishmania major* lesions in BALB/c mice infected with stationary-phase promastigotes cultured at pH 7·0 (■——■) and 5·5 (●——●). Each group contained 12 animals. The bars represent 1 standard error of the mean.

liver while those infected with promastigotes cultured at pH 7·0 had approximately 2.8×10^6 amastigotes/liver.

Stationary-phase promastigotes of *L. mexicana*, *L. major* and *L. braziliensis* cultured at pH 7·0 and 5·5 were used to infect 2 groups of 12 BALB/c mice for each species. Mice were examined once a week for 10 weeks and the sizes of cutaneous lesions were measured. In all groups for each of the 3 species, lesion development and size was faster in mice inoculated with promastigotes cultured at pH 5·5 than in those inoculated with promastigotes cultured at pH 7·0 (Figs 2–4). In the case of *L. mexicana* and *L. major*, a superficial, non-ulcerating papule appeared first. This developed into a nodule with a
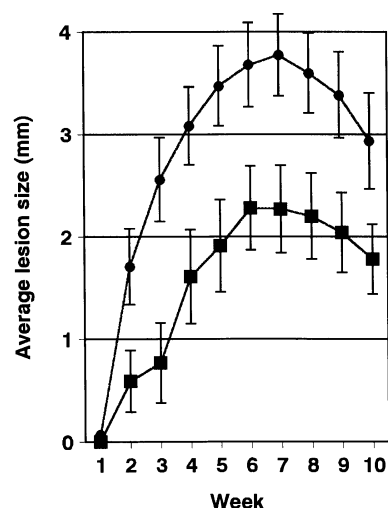
Fig. 4. Development of *Leishmania braziliensis* lesions in BALB/c mice infected with stationary-phase promastigotes cultured at pH 7·0 (■———■) and 5·5 (●———●). Each group contained 12 animals. The bars represent 1 standard error of the mean.

larger border and deeper centre. A few mice inoculated with *L. major* had centrally ulcerated nodules during the last 2 weeks of the experiment. In the case of *L. braziliensis*, lesions appeared as small hairless patches of rough skin at the site of inoculation. Even though such lesions were not very distinctive, they were larger and their appearance was earlier in mice inoculated with promastigotes cultured at pH 5·5 compared to mice inoculated with promastigotes cultured at pH 7·0.

DISCUSSION

When cultures of *Leishmania* promastigotes reach stationary phase, a proportion of the population may differentiate into metacyclic promastigotes (Sacks, 1989). This proportion depends upon, amongst other factors, the length of time the promastigotes have been maintained *in vitro* (da Silva & Sacks, 1987). In a previous study, we showed that cultivation of recently transformed (2–4 days) *L. mexicana* promastigotes in Schneider's *Drosophila* medium supplemented with 20% heat-inactivated FCS at acidic pH produced a homogeneous stationary-phase population of metacyclic-like promastigotes. However, cultivation of such promastigotes to stationary phase at pH 7·0 yielded a mixed population of metacyclic and other forms (Bates & Tetley, 1993).

In this study we were able to stimulate metacyclogenesis in promastigotes of *L. braziliensis*, *L. donovani*, and *L. major* using similar methodology. Culture-derived metacyclic promastigotes were defined by possession of a short ($\leqslant 8\ \mu$m), narrow ($\leqslant 1\cdot5\ \mu$m) cell body and a long flagellum twice or more the length of the cell body. These metacyclic promastigotes appeared as early as day 2 during the exponential phase in acidic cultures (pH 5·5) and by

the stationary phase, 49–78% of promastigotes were metacyclic forms, depending on the species. In contrast, only 10–29% of promastigotes cultured originally at pH 7·0 differentiated to metacyclic promastigotes by the stationary phase. Although the percentage of metacyclic promastigotes produced varied between the different species, consistency in producing similar percentages of metacyclic promastigotes was achieved in repeated experiments (not shown). However, we cannot exclude strain as opposed to species differences as a factor in the variation observed.

One of the defence components of the mammalian host against parasitic protozoa is the complement system. Therefore, for promastigotes to be able to establish a *Leishmania* infection, they have to be resistant to complement-mediated lysis. Metacyclic promastigotes are not only resistant to complement-mediated lysis, but in addition they use complement components as ligands for binding and entry into macrophages (Alexander & Russell, 1992). In this study stationary-phase promastigotes grown in acidic pH were more resistant to complement-mediated lysis than those grown at neutral pH. A 4 to 16-fold increase in resistance was observed among the 4 *Leishmania* species studied. This is consistent with previous reports of metacyclogenesis *in vitro* (Franke *et al*. 1985; Howard, Sayers & Miles, 1987; Wozencraft & Blackwell, 1987; Bandyopadhyay *et al*. 1991, Almeida *et al*. 1993; Bates & Tetley, 1993).

Another criterion that has been used to characterize metacyclic promastigotes is their infectivity *in vivo*. *Leishmania* parasites have been shown to infect several models (reviewed by Hommel *et al*. 1995), although none of these models completely accurately reproduces what happens in humans. In this study BALB/c mice were used to investigate the difference between the infectivity of stationary-phase *L. donovani* promastigotes cultured at pH 5·5 and those cultured at pH 7·0. Two weeks after inoculation, there was a statistically significant difference between the 2 types of inocula. Metacyclic promastigotes obtained from the stationary phase of acidic cultures were approximately 20 times more infective than promastigotes obtained in the stationary phase from neutral cultures.

With *L. mexicana* and *L. major*, and using the same inoculum size, mice infected with stationary-phase promastigotes from acidic cultures developed cutaneous lesions earlier than those infected with stationary-phase promastigotes from neutral cultures. The sizes of lesions increased consistently as the experiment progressed. When the average lesion diameter was plotted against time the standard error bar of the 2 curves never overlapped. This demonstrated that the lesion development in one group was consistently greater than that of the other group.

L. braziliensis lesions appeared as small hairless rough skin at the site of inoculation, even though a

larger inoculum was used. However, the sizes of lesions were larger and their appearance was earlier in mice inoculated with promastigotes cultured at pH 5·5.

In this study we have used acidic conditions to stimulate metacyclogenesis *in vitro*. Whether this also occurs *in vivo* is a matter for speculation, but 2 observations suggest that it may do so. During growth *in vitro* promastigotes are normally seen to acidify the culture medium in which they are growing. The densities in such cultures are 10^7–10^8 cells/ml. *In vivo* promastigotes can achieve very high densities within the sandfly midgut, forming a solid mass of parasites embedded in a gel-like matrix in the anterior midgut, something equivalent to 10^9–10^{10} cells/ml. Thus it seems quite possible that this density of parasites could case a fall in pH. The second observation is some recent measurements of pH in the gut of *Lutzomyia longipalpis* (Tang, Anez & Bates, 1997), which found a neutral to slightly acidic pH in the anterior midgut, compared to a more alkaline pH in the posterior midgut. These studies were performed on uninfected sandflies only, as thus far it has proved technically difficult to perform the same experiments on infected flies.

In conclusion, this study has shown that it is possible to stimulate the production of metacyclic-like promastigotes of *L. braziliensis*, *L. donovani*, *L. major* and *L. mexicana in vitro* by culturing at pH 5·5.

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