Axenic cultivation and characterization of *Leishmania mexicana* amastigote-like forms

P. A. BATES[1], C. D. ROBERTSON[1], L. TETLEY[2] *and* G. H. COOMBS[1]

[1] *Laboratory for Biochemical Parasitology, Department of Zoology, University of Glasgow, Glasgow G12 8QQ*
[2] *Electron Microscopy Centre, Department of Zoology, University of Glasgow, Glasgow G12 8QQ*

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SUMMARY

A new method is described which has made possible the long-term axenic cultivation of *Leishmania mexicana* amastigote-like forms in Schneider's *Drosophila* medium supplemented with 20 % (v/v) foetal calf serum. Unlike previous methods, it utilizes direct culture of parasites obtained from the lesions of infected animals rather than adaptation of promastigotes *in vitro*. Ultrastructural (possession of megasomes), biochemical (cysteine proteinase activity and gelatin SDS–PAGE banding pattern) and infectivity (*in vivo*) data are presented which show the close similarity of the cultured forms to lesion amastigotes. The axenically cultured forms grew optimally at a temperature of 32–33 °C, providing further evidence for their amastigote nature. It was found that adjustment of the pH of the growth medium to 5·4 was required in order to retain the amastigote morphology of the cultured parasites. This supports the notion that leishmanial amastigotes are acidophiles.

Key words: *Leishmania mexicana*, axenic culture, amastigotes, megasomes, cysteine proteinase, infectivity.

INTRODUCTION

Leishmania parasites exist as amastigotes within the phagolysosomes of macrophages in their mammalian hosts (Alexander & Vickerman, 1975; Chang & Dwyer, 1976). The mechanisms which amastigotes employ to survive in this apparently inhospitable environment are only partly understood, due in part to the difficulty of conducting biochemical studies directly on parasites within their host cells. Isolation of amastigotes from infected animals (Childs, McRoberts & Foster, 1976; Brazil, 1978; Infante *et al.* 1980; Hart, Vickerman & Coombs, 1981*a*; Saraiva *et al.* 1983; Meade *et al.* 1984; Glaser *et al.* 1990) or from macrophage cultures infected *in vitro* (Chang & Dwyer, 1978; Berens & Marr, 1979; Berman, Dwyer & Wyler, 1979; Chang, 1980*a*; Looker *et al.* 1986; Martinez, Looker & Marr, 1988) is often labour-intensive and/or provides insufficient material for biochemical studies. Also such methods leave doubts concerning the purity of the amastigote preparation and the presence in it of adsorbed host components. The alternative, axenic *in vitro* cultivation of amastigote-like forms, has proved relatively difficult to achieve when compared with promastigote culture, but has been reported for *L. pifanoi*, *L. panamensis*, *L. braziliensis* and *L. donovani* (Pan, 1984; Eperon & McMahon-Pratt, 1989*a*; Doyle *et al.* 1991). These difficulties have determined that the biochemical characterization of *Leishmania* has centred on cultured extracellular promastigotes as a model system, and investigations on amastigotes have lagged somewhat behind. In this paper we present a new method for the axenic cultivation of *Leishmania mexicana* in a form that from morphological, biochemical and biological data appears similar to lesion amastigotes. Evidence is also presented that, compared with promastigotes, *L. mexicana* amastigotes require an elevated temperature and a relatively acidic environment for growth. The use of this system should facilitate studies on the biology of *L. mexicana* amastigotes.

MATERIALS AND METHODS

In vitro *culture*

The following protocol was developed (see Results section) and used routinely for the axenic culture of *L. mexicana* amastigotes. Infections of *L. mexicana* (MNYC/BZ/62/M379) were maintained in female CBA mice as previously described (Hart *et al.* 1981*a*). Mice with medium-sized unruptured lesions (10–15 mm diameter) were selected as a source of amastigotes, so as to minimize the danger of contamination with unwanted organisms. Mice were killed by terminal anaesthesia and rinsed with 70 % (v/v) ethanol to sterilize the skin. The lesions were excised aseptically in a laminar flow hood and all subsequent manipulations performed under sterile conditions. A single lesion was able to provide sufficient amastigotes on each occasion that cultures were established.

The lesion was gently homogenized in 10 ml of complete growth medium (see below) using a sterile 10 ml syringe plunger to push the tissue through a fine wire mesh in a Petri dish. The resulting crude homogenate usually contained 10^8–10^9 free amastigotes/ml and was centrifuged at 1000 ***g*** for 5 min at ambient temperature (22 °C). The pellet containing large clumps of amastigotes, erythrocytes and other host cells was discarded, and the supernatant medium containing 10^7–10^8 amastigotes/ml was retained and further centrifuged at 2000 ***g*** for 5 min at 22 °C. The resulting supernatant medium containing host cell debris was discarded and the amastigote pellet resuspended in 10 ml of fresh growth medium. Cultures were initiated by subinoculation of the amastigote preparation into 10 ml of fresh growth medium, to a final density of 5×10^5/ml, in 25 cm^2 tissue-culture flasks. Cultures were maintained stationary in the sealed flasks, on their side at 32–33 °C with an initial gas phase of air, and subpassaged every 5–6 days at the late log/early stationary phase of growth (see Results section). This was achieved by passing cultured amastigotes through a sterile 26-gauge needle to break up any large clumps of cells, and initiating new cultures at $1–5 \times 10^5$ cells/ml as desired.

Growth medium consisted of Schneider's *Drosophila* Medium (Gibco Ltd, Paisley, Scotland) supplemented with 20 % (v/v) foetal calf serum (Gibco) and 25 μg gentamicin sulphate/ml. A variety of pH conditions were tested initially (see Results section), and these experiments established that for routine culturing the optimal initial pH was $5{\cdot}4 \pm 0{\cdot}1$, obtained using 1 M HCl.

Promastigotes were obtained by transformation of lesion amastigotes and cultured in HOMEM supplemented with 10 % foetal calf serum and 25 μg gentamicin sulphate/ml as previously described (Hart, Vickerman & Coombs, 1981*b*; Mallinson & Coombs, 1989).

Cell counting was performed using Improved Neubauer haemocytometers under phase-contrast microscopy. Amastigotes isolated from lesions and those cultured *in vitro* had a tendency to clump together. Such clumps were disrupted for the purposes of counting by passing the cells 3 times through a 26-gauge needle.

Ultrastructure

Transmission electron microscopy of axenic amastigotes was performed as previously described for lesion amastigotes (Coombs *et al.* 1986).

Proteinases

Enzyme assays and gelatin SDS–PAGE were performed as previously described (Robertson & Coombs, 1990).

Infectivity

The infectivity of lesion amastigotes, axenic amastigotes and promastigotes was determined essentially as described by Alexander (1988). Lesion amastigotes were used immediately after isolation. Axenic amastigotes were taken from day 5–6 cultures and promastigotes from day 7 cultures, i.e. at stationary phase when the numbers of metacyclic promastigotes are maximal (Mallinson & Coombs, 1986, 1989). To obviate any possible complicating effects of the length of *in vitro* culture on the infectivity of parasites, matched cultures of amastigotes and promastigotes which had been cultured *in vitro* for identical periods and within 7 subpassages from an infected animal were used to infect mice. For each form, 5×10^6 parasites were inoculated subcutaneously into the shaven rump of each 5- to 6-week-old female CBA mouse used. After 4 weeks and thereafter at 2-weekly intervals until 20 weeks, mice were shaved, examined and the diameter of lesions determined using a Mitutoyo micrometer. Infectivity was calculated as the mean lesion diameter, including all animals in a group and treating those without lesions as possessing a lesion of 0·0 diameter. We do not assume that the data are normally distributed, and therefore a non-parametric test, the Wilcoxon–Mann–Whitney, was used to determine the statistical significance of any observed differences in lesion diameter between groups of mice.

RESULTS

In vitro *culture*

Initial experiments were conducted to optimize conditions for axenic cultivation of amastigotes. Three factors were varied: medium composition, temperature and pH. As a result of these experiments, described fully below, a standard growth medium comprising Schneider's *Drosophila* medium supplemented with 20 % (v/v) foetal calf serum and with a pH of 5·4 was developed. Establishment of cultures used a partially purified preparation of amastigotes (see Materials and Methods section), which contained low numbers of erythrocytes and other host cells. However, their presence did not affect the viability of the amastigotes as assessed by their ability to grow *in vitro*. Such contaminants were degraded and undetectable within 24 h of culture.

A typical growth curve for axenic amastigotes of *L. mexicana* cultured in standard growth medium is shown in Fig. 1. In common with other microorganisms cultured *in vitro*, a logarithmic growth phase was observed, followed by a period of slower growth, a stationary phase and eventually cell death leading to a decrease in density. Typically, no lag phase was observed. Generally, doubling times varied between 18 and 22 h and the final cell density was in the range 4 to 6×10^7/ml. Occasionally, faster

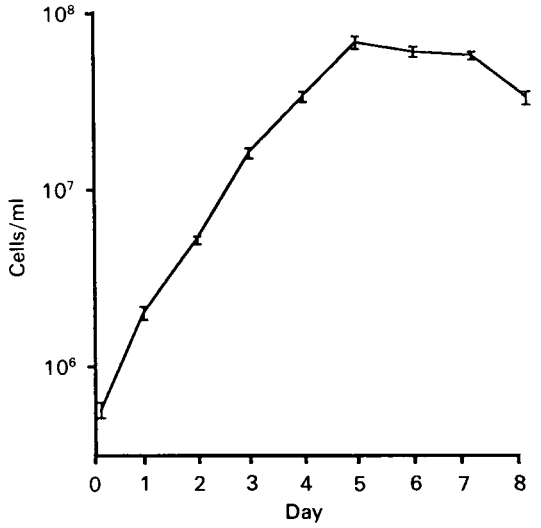

Fig. 1. Typical growth curve for axenic amastigotes of *Leishmania mexicana*. A culture was initiated at 5×10^5 cells/ml and samples taken on consecutive days for cell counting. The vertical bars represent one standard deviation from the mean value.

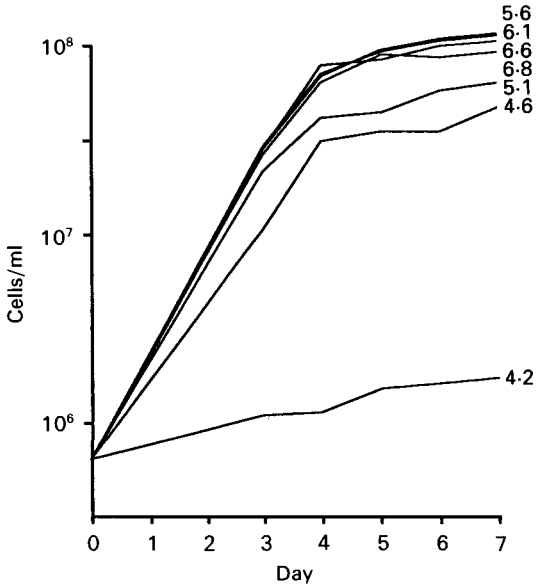

Fig. 2. The effects of varying the pH of the medium on the growth of axenic amastigotes. Cultures of axenic amastigotes were initiated using the same pool of cells and at the same initial density into standard growth medium which had been adjusted to different pH values. Counts were made on days 3–7 to assess the effect on growth. Error bars are omitted for clarity but were similar to those in Fig. 1. The pH values of the media for each culture used in this experiment were as indicated on the figure.

growth rates were observed, with doubling times of 14–18 h, and/or final cell densities of up to 10^8/ml. Cultures were subpassaged every 5–6 days. After day 8–9 cell numbers began to fall, unless cultures were supplemented with fresh medium. Although individual cells were present in cultures, many of the axenic amastigotes grew in clumps of 4 to about 50 cells, but this did not have an adverse effect on their growth rate. Using this methodology it has been possible to maintain cultures continuously for a period of 10 months, involving 60 subpassages in which growth was essentially the same, at which time cultures were discontinued. However, all experiments subsequently described were performed on amastigotes which had been in culture for no more than 10 subpassages.

Two different media formulations, HOMEM (Berens, Brun & Krassner, 1976) and Schneider's *Drosophila* medium (Hendricks, Wood & Hadjuk, 1978), supplemented with foetal calf serum to either 10 or 20% (v/v) were tested for the ability to support growth of amastigotes *in vitro*. All media contained 25 μg gentamicin sulphate/ml. HOMEM supplemented with 10% foetal calf serum is used routinely in our laboratory for the axenic cultivation of promastigotes (Mallinson & Coombs, 1989). All these media were able to support growth of amastigotes to some extent, but the best results were obtained using Schneider's *Drosophila* medium supplemented with 20% serum. This was judged by a faster rate of multiplication and higher final cell density when compared with the other media combinations (data not shown).

Temperature was also varied between 28 and 37 °C. Optimum conditions, as judged from cell morphology and growth rate, were at 32–33 °C. At temperatures of 30 °C and below, amastigotes tended to transform and grow as promastigotes or intermediate ellipsoid forms, some of which possessed short flagella. At higher temperatures amastigote morphology was retained. Cultures could grow at 34 °C, albeit more slowly, but at 35 °C and above growth was inhibited and the cells died (data not shown).

Initially, all culture attempts utilized media which were close to neutral pH, i.e. pH 7·6 for HOMEM (as originally specified, Berens *et al.* 1976) and pH 6·9–7·1 for Schneider's (as supplied by Gibco). However, it was observed that cultivation of *L. mexicana* amastigotes under these conditions led to the appearance of promastigotes and other forms intermediate in morphology, much as occurred during cultivation at lower temperatures. The effects of lowering the pH of the various media were investigated, because amastigotes reside in the phagolysosomal system of macrophages *in vivo*. A variety of pH conditions were tested, and the results of one such experiment in which the values were varied between pH 4·2 and 6·8 are shown in Fig. 2. In this experiment, no inhibition of growth was seen at pH 5·6 and above. Lower but significant growth was achieved at pH 5·1 and 4·6, but at lower pH values the rate of multiplication and final cell density were increasingly depressed. At pH 6·6 and above, promastigote and intermediate forms were observed.

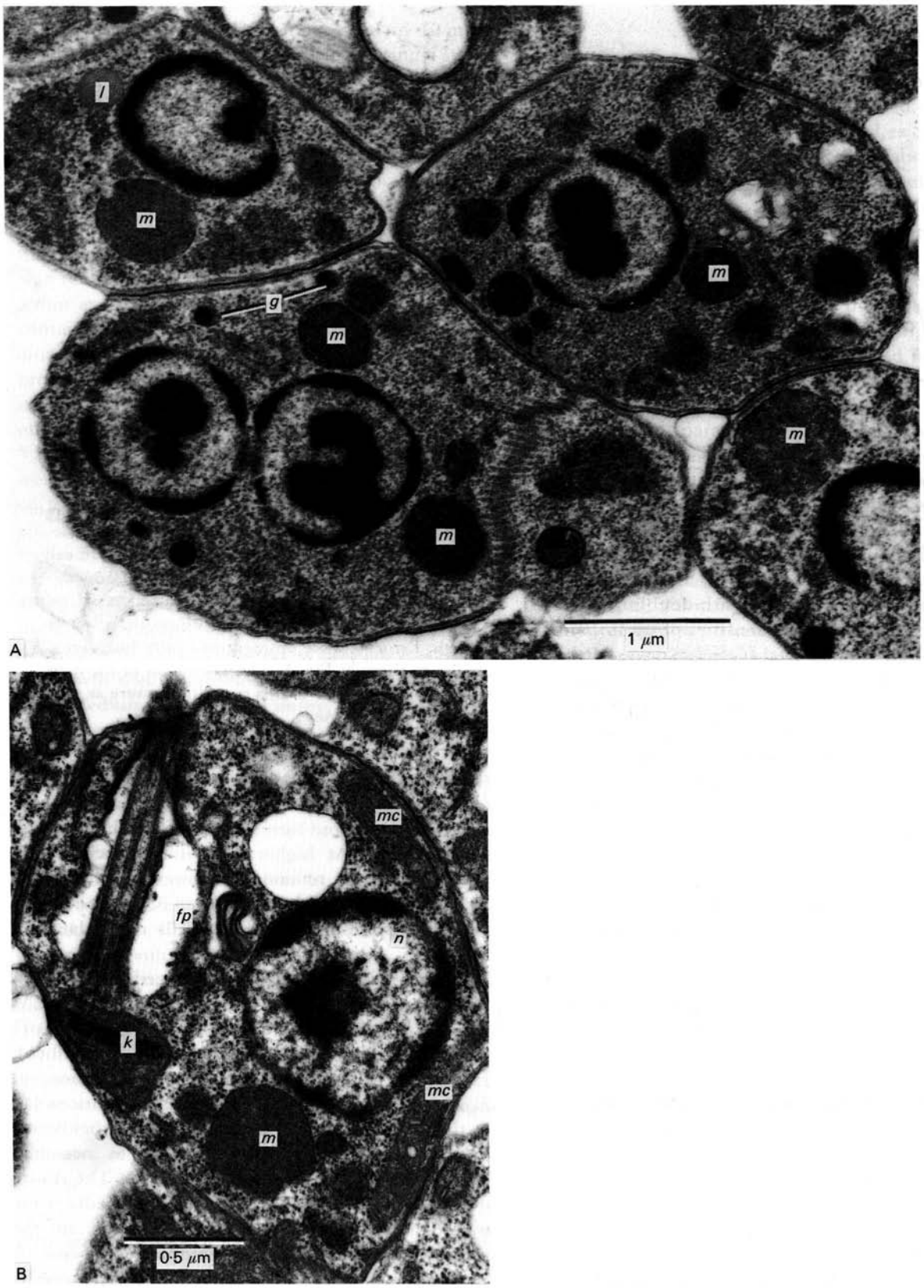

Fig. 3. Morphology by electron microscopy of axenic amastigotes, harvested at day 3 of culture (log phase). (A) Low magnification showing several axenic amastigotes. Note the dividing form with 2 nuclei, megasomes, glycosomes and a lipid droplet. For clarity some of the organelles have been left unlabelled. (B) Longitudinal section through an individual axenic amastigote showing the non-emergent flagellum without a paraxial rod, the kinetoplast-mitochondrion and a large megasome next to the nucleus. *fp*, Flagellar pocket; *g*, glycosome; *k*, kinetoplast; *l*, lipid droplet; *m*, megasome; *mc*, mitochondrion; *n*, nucleus.

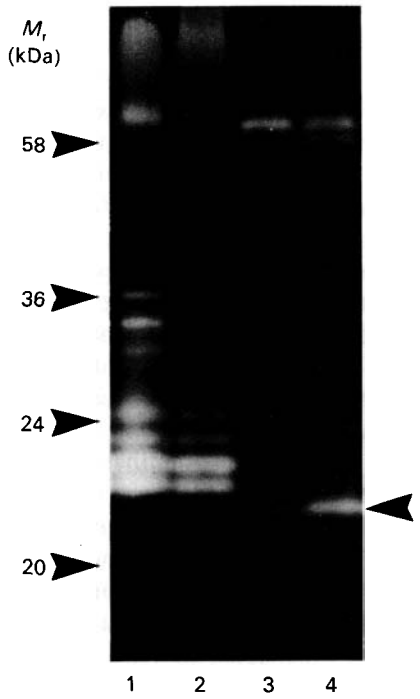

Fig. 4. Gelatin SDS–PAGE analysis of various forms of *Leishmania mexicana*. Lane 1, axenic amastigotes; Lane 2, lesion amastigotes; Lane 3, log-phase promastigotes; Lane 4, stationary-phase promastigotes. Each lane was loaded with 30 μg of total cell protein.

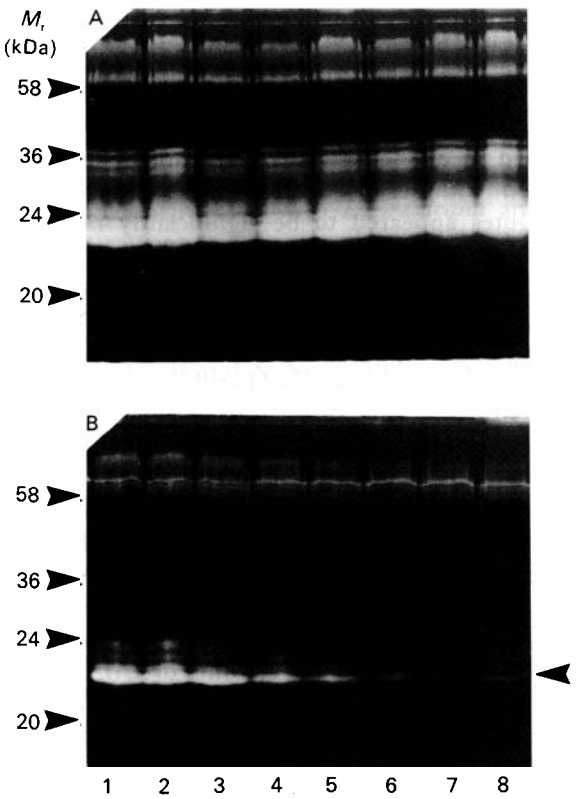

Fig. 5. Gelatin SDS–PAGE analysis of axenic amastigotes (A) and stationary-phase promastigotes (B) taken from different subpassages after establishment of *in vitro* cultures. In each gel, Lanes 1–8 represent samples taken from subpassages 1–8, respectively. Each lane was loaded with 15 μg of total cell protein.

From such experiments it was established that for retention of amastigote morphology and high growth rate the external pH needed to be between 5·3 and 6·4. Cultivation outside these limits led to aberrant morphology and/or poorer growth. It was noted that cultivation and growth of amastigotes under the conditions tested led to a slight acidification of the culture medium, typically 0·1–0·3 pH units by day 7.

Morphology

The first criterion employed to assess the relationship between axenic and lesion amastigotes was ultrastructure, as revealed by transmission electron microscopy (Fig. 3). Axenic amastigotes possessed general features characteristic of *Leishmania* parasites as indicated, but also features specifically found in amastigotes: an ovoid shape of 3–5 μm on the major axis, a short non-emergent flagellum and the absence of a paraxial rod (Pan & Pan, 1986; Eperon & McMahon-Pratt, 1989*a*). Significantly, they also contained megasomes, the characteristic lysosomes of *L. mexicana* amastigotes, which are not found in promastigotes (Alexander & Vickerman, 1975; Coombs *et al.* 1986). These were identified by their electron density and the occurrence of a bounding membrane comparable in thickness to the plasma membrane (10 nm), thus distinguishing them from glycosomes (bounding membrane 7 nm) and lipid droplets (no membrane).

Proteinases

The second criterion employed was the possession of cysteine proteinases. Axenic amastigotes and promastigotes were harvested after 5–6 days in culture, and proteinase activities in whole-cell homogenates determined using the substrate *N*-benzoyl-prolyl-phenylalanyl-arginyl-*p*-nitroanilide (BzPFRNan). The mean activity detected in late log phase/early stationary phase axenic amastigotes was $49{\cdot}3 \pm 10{\cdot}5$ (2 S.E., $n = 14$) nmol/min per mg protein, significantly higher than that found in equivalent promastigotes, $11{\cdot}2 + 3{\cdot}9$ (2 S.E., $n = 14$). These values compare with 100 nmol/min/mg protein determined for homogenates of purified lesion amastigotes. Approximately 50% of the activity detected in axenic amastigotes using this substrate was inhibitable by E-64, indicating that it was due to cysteine proteinases. The occurrence of cysteine proteinases was investigated in more detail using gelatin SDS–PAGE gel analyses (Fig. 4). The pattern of bands seen in axenic amastigotes was very similar to that observed in lesion amastigotes in terms of apparent molecular weight and relative intensity of individual bands. One quantitative difference was that more intense proteinase bands in the molecular weight range 30–36 kDa were apparent in the former samples. Little could be detected using log-phase

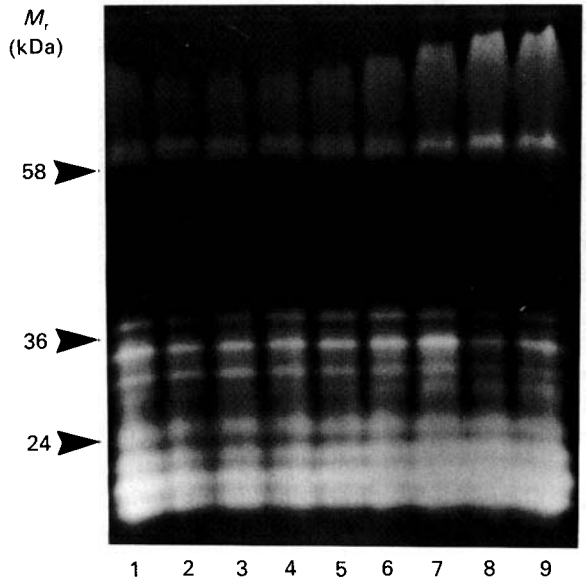

Fig. 6. Gelatin SDS–PAGE analysis of proteinases during the growth cycle of *Leishmania mexicana* axenic amastigotes. A culture was initiated at 5×10^5 cells/ml with axenic amastigotes (Lane 1) and samples removed on consecutive days of *in vitro* culture (Lanes 2–9). Each lane was loaded with 15 μg of total cell protein.

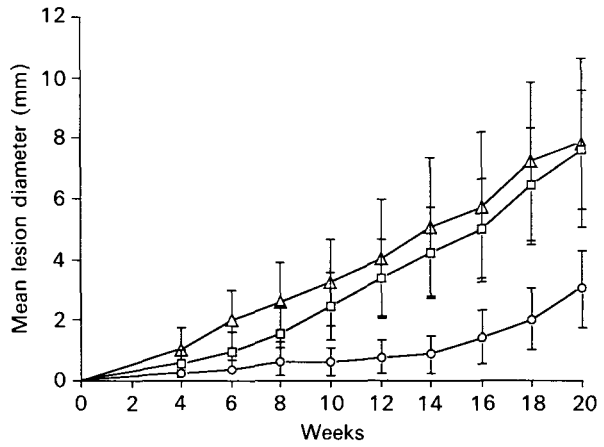

Fig. 7. A comparison of the infectivity of lesion amastigotes (△—△), axenic amastigotes (□—□) and stationary-phase promastigotes (○—○) in groups of 24, 30 and 30 female CBA mice, respectively. The bars represent two standard errors from the mean lesion diameter.

promastigotes, but enzymes were detected in samples of stationary-phase promastigotes. A lower molecular weight cysteine proteinase characteristically found in the latter (arrowed, Fig. 4) was apparently absent from either form of amastigote.

The occurrence of cysteine proteinases as a function of length of time in culture was also investigated using gelatin SDS–PAGE. Fig. 5 shows an analysis of samples of both axenic amastigotes (panel A) and promastigotes (panel B) taken at subpassages 1–8 after establishment in culture from lesion amastigotes. The pattern observed in amastigotes was consistent, and of greater intensity than that observed in the equivalent promastigote cultures of identical age. This result indicates that for this biochemical marker the difference between axenic amastigotes and promastigotes is preserved for at least 8 subpassages *in vitro*. Interestingly, the cysteine proteinases characteristic of promastigotes decreased during serial subpassage *in vitro* (arrowed, Fig. 5).

Cysteine proteinases were also examined during the growth cycle of amastigotes *in vitro*. Fig. 6 shows an example of a gelatin SDS–PAGE analysis of axenic amastigotes taken from consecutive days of *in vitro* culture. The intensity of the banding pattern observed indicated that during the initial phase of logarithmic growth (lanes 1–3) the proteinase activity per cell fell, then remained relatively constant as the cells continued to divide (lanes 3–5), increased during the late log phase (lanes 5–7) and finally reached a maximal level at stationary phase (lanes 8–9). Similar results were obtained using enzyme assays with BzPFRNan (data not shown).

Infectivity

The third criterion employed was the ability of axenic amastigotes to produce lesions in experimentally infected mice as compared with both lesion amastigotes and stationary-phase promastigotes. Fig. 7 shows the results of such an experiment. Lesion amastigotes were highly infectious, producing lesions within 4 weeks which increased in size steadily during the course of the experiment. Axenic amastigotes produced lesions of similar size, which increased in diameter at a rate comparable to that observed with lesion amastigotes. Both forms of amastigotes appeared to be significantly more infective than stationary-phase promastigotes, which produced smaller lesions increasing in size at a slower rate. Statistical analysis showed that at 8 weeks the difference observed between axenic amastigotes and promastigotes could still be explained by chance ($0{\cdot}1 > P > 0{\cdot}05$). However, the results at 10 weeks and thereafter indicated that axenic amastigotes were significantly more infective than promastigotes ($P < 0{\cdot}01$ for all these time-points). In contrast, no statistically significant difference was observed between axenic amastigotes and lesion amastigotes for any of the time points ($P > 0{\cdot}25$). Lesion amastigotes and axenic amastigotes were both infective for the J774 mouse cell line or CBA mouse peritoneal exudate cells when tested *in vitro*, and produced the characteristically large parasitophorous vacuoles associated with *L. mexicana* (data not shown).

DISCUSSION

The general pattern of growth observed in cultures of *L. mexicana* axenic amastigotes was similar to that reported for *in vitro* cultures of amastigotes of other

species (Pan, 1984; Eperon & McMahon-Pratt, 1989*a*). Doubling times were normally in the range 18–22 h, and thus very similar to the 17·5 h reported for *L. mexicana* amastigotes multiplying in human macrophages *in vitro* (Doyle *et al.* 1989).

Since metacyclic promastigotes experience an increase in temperature upon injection into the mammalian host, many investigators have studied the morphological and biochemical responses of promastigotes cultured *in vitro* to elevated temperatures (see Shapira, McEwen & Jaffe (1988) and Smejkal, Wolff & Olenick (1988) for references). Such treatments often resulted in rounding up and adoption of an amastigote-like morphology. However, the resulting cells rarely survived more than a few days, and it is thus doubtful that these were true amastigotes. In two cases, however, it proved possible, by manipulation of the temperature in gradually increasing increments, to achieve transformation of cultured promastigotes and subsequent serial passage of parasites which were convincingly similar to lesion amastigotes. This was first achieved for *L. pifanoi* by adaptation to 33 or 35 °C (Pan, 1984) and latterly for *L. panamensis* at 32 °C, *L. braziliensis* at 28 °C (Eperon & McMahon-Pratt, 1989*a*), and *L. donovani* at 37 °C (Doyle *et al.* 1991). A different approach was used in our study. Rather than attempting to adapt promastigotes, amastigotes were isolated from lesions and directly cultured at elevated temperatures. Based on growth rate and morphology data, the optimum temperature was found to be 32–33 °C. This is consistent with *L. mexicana* infection resulting in cutaneous rather than visceral leishmaniasis. *In vitro* cultures of macrophages infected with *L. mexicana* and other species causing cutaneous disease are maintained at comparable or slightly higher temperatures of 33–35 °C (Chang, 1980*a*; Berman & Neva, 1981; Hart *et al.* 1981*a*; Biegel, Topper & Rabinovitch, 1983; Sacks, Barral & Neva, 1983; Scott, James & Sher, 1985; Martinez *et al.* 1988). In these studies with macrophages, 37 °C was found to be an unsuitable temperature for growth of *L. mexicana*. Our results suggest that this was due to the inability of amastigotes themselves to withstand this temperature, rather than an effect on the macrophage–amastigote interaction.

The effects of pH on *Leishmania* parasites in relation to the growth of amastigote-like forms have not been reported previously. Our initial attempts to culture *L. mexicana* amastigotes axenically in neutral media were prompted by previous reports (Pan, 1984; Eperon & McMahon Pratt, 1989*a*; Doyle *et al.* 1991). However, we found that culturing at neutral pH led to the progressive appearance of promastigotes and intermediate forms in cultures. Manipulation of the pH conditions in axenic cultures demonstrated that retention of amastigote morphology required a pH below 6·4. pH 5·4 was selected as the standard condition, although growth at slower rates was possible down to pH 4·5. These responses to pH support current views on conditions within the parasitophorous vacuoles of infected macrophages. The vacuole results from phagosome–lysosome fusion (Alexander & Vickerman, 1975; Chang & Dwyer, 1976) and differentiating promastigotes and amastigotes are therefore exposed to acidic conditions. Until recently, direct measurements of the pH of the parasitophorous vacuole have been preliminary in nature (Chang, 1980*b*; Rivas & Chang, 1983). However, in a recent more detailed study using *L. amazonensis*, which develops in a large vacuole similar to *L. mexicana*, Antoine *et al.* (1990) concluded that the pH of the parasitophorous vacuole was between 4·74 and 5·26. These values were lower than the range of 5·17 to 5·48 reported by the same authors for lysosomes in uninfected control macrophages. Our observation that culture of axenic amastigotes led to acidification of the culture medium by 0·1–0·3 pH units to pH 5·3–5·1 suggests that the amastigotes themselves may directly acidify the vacuole. A similar effect occurs in promastigote cultures as a result of the release of various organic acids (Marr, 1980). In further support of the notion that amastigotes are acidophiles, it has been shown that *L. donovani* amastigotes are metabolically more active at acidic pH, and also able to regulate their internal pH close to neutral under these conditions (Mukkada *et al.* 1985; Glaser *et al.* 1988). It has also been noted that growth of various *Leishmania* promastigotes at acidic pH induces synthesis of an amastigote-specific protein (Zilberstein *et al.* 1991), observations which are supported by the current report.

Various criteria have been applied to determine the similarity or otherwise of *in vitro* amastigote-like forms to lesion amastigotes. At the ultrastructural level, amastigotes display certain characteristic features in addition to those in common with promastigotes and other trypanosomatids (Pan & Pan, 1986; Eperon & McMahon-Pratt, 1989*a*). Such features were also found with axenic amastigotes of *L. mexicana*. Futher, axenic amastigotes were shown to possess megasomes, organelles unique to amastigotes of *L. mexicana* and related species, and not found in promastigotes. Qualitatively, the cultured forms are clearly amastigote-like. The number of megasomes has not been directly quantified, but there appeared to be fewer per cell than in lesion amastigotes as judged from overall appearance (Alexander & Vickerman, 1975; Coombs *et al.* 1986). This may simply reflect the relatively rapid growth of axenic amastigotes *in vitro* as compared with their *in vivo* counterparts, with the rate of organelle biosynthesis initially lagging behind the rate of cell growth. This was supported by the results of analysing the cysteine proteinase activity of cells harvested on consecutive days of *in vitro* culture.

These enzymes probably reside in the megasomes (Pupkis, Tetley & Coombs, 1986), and it was noted that, after falling slightly during the early log phase, the banding pattern became more intense in stationary-phase cells. Therefore, in this respect stationary-phase axenic amastigotes most closely resembled the lesion amastigotes.

Various biochemical criteria have been used to compare axenically cultured and lesion amastigotes of *L. pifanoi* (Pan, McMahon-Pratt & Honigberg, 1984; Pan, 1986; Pan & McMahon-Pratt, 1988; Rainey *et al.* 1991), *L. panamensis* and *L. braziliensis* (Eperon & McMahon-Pratt, 1989*b*). In our study we examined *L. mexicana* axenic amastigotes for cysteine proteinases. Proteinases in general, and these enzymes in particular, were found at the highest specific activity in lesion amastigotes, at somewhat lower levels in stationary-phase promastigotes and at very low levels in multiplicative promastigotes (North & Coombs, 1981; Coombs, 1982; Pupkis & Coombs, 1984; Pupkis *et al.* 1986; Lockwood *et al.* 1987). Using BzPFRNan, lesion amastigotes were found to possess approximately 10 times and axenic amastigotes 5 times the level of proteinase activity of stationary-phase promastigotes. This result is similar to those of previous comparisons of lesion amastigotes and promastigotes (Coombs, 1982; Pupkis & Coombs, 1984; Pupkis *et al.* 1986). It also suggests that proteinase activity in axenic amastigotes is slightly lower than in lesion amastigotes, in agreement with the apparently lower numbers of megasomes (see above).

A more detailed analysis of cysteine proteinases was performed using gelatin SDS–PAGE, where a prominent group of bands in the apparent molecular-weight range 20–25 kDa have been demonstrated in *L. mexicana* (Lockwood *et al.* 1987; Robertson & Coombs, 1990; Coombs, Robertson & Mottram, 1991). The pattern observed in lesion amastigotes and axenic amastigotes was qualitatively identical in this region. However, there appeared to be more intense banding in the molecular weight range 30–36 kDa in samples from axenic amastigotes. Whether these have any relationship to the lower molecular weight enzymes, precursors for example, is unknown. Differences in the apparent molecular weights of cysteine proteinases in promastigotes and amastigotes have been noted before (North & Coombs, 1981; Coombs *et al.* 1991) and here we demonstrated that a low molecular weight enzyme found characteristically in stationary-phase promastigotes was undetectable in both axenic and lesion amastigotes.

The third criterion, and a key biological feature of axenic amastigotes, is that they should show infectivity *in vivo* similar to that of lesion amastigotes, which have been reported to be generally more infective than cultured promastigotes. This criterion has been partly met for *L. pifanoi* axenic amastigote-like forms, which were compared with amastigotes derived from *in vitro*-infected J774 macrophage cultures (Pan & Honigberg, 1985). These two forms were shown to be equally infective to the J774 mouse cell line or to hamsters via footpad inoculation. In the current study we applied a more stringent test, comparing axenic amastigotes with those isolated from lesions of infected animals for their ability to cause rump lesions in naïve mice. The results indicated that *L. mexicana* axenic amastigotes were similar in infectivity to those isolated directly from infected animals, and both of these were significantly more infective than promastigotes.

In summary, we have reported for the first time a successful method for the direct axenic cultivation of leishmanial amastigotes. For each of the criteria we have employed, the data presented demonstrate that the cultured forms are indeed amastigote-like and not rounded aflagellate promastigotes. Not surprisingly, some quantitative differences were observed in comparison with lesion amastigotes, and these are the subject of further investigation. Similar differences have also been observed with other species (Rainey *et al.* 1991). Such differences may simply arise from different growth rates, but could indicate that some further refinement of culture conditions would be beneficial. It should be borne in mind, however, that amastigotes in animals may themselves not all be identical, varying for instance with their location in the lesion. Nevertheless, as long as the caveats of any *in vitro* system are borne in mind, the method described should provide an excellent model enabling further biochemical studies of *L. mexicana* amastigotes including certain types of work which are otherwise impossible to conduct.

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REFERENCES

ALEXANDER, J. (1988). Sex differences and cross-immunity in DBA/2 mice infected with *Leishmania mexicana* and *L. major*. *Parasitology* **96**, 297–302.

ALEXANDER, J. & VICKERMAN, K. (1975). Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of *Leishmania mexicana*-infected macrophages. *Journal of Protozoology* **22**, 502–8.

ANTOINE, J.-C., PRINA, E., JOUANNE, C. & BONGRAND, P. (1990). Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. *Infection and Immunity* **58**, 779–87.

BERENS, R. L. & MARR, J. J. (1979). Growth of *Leishmania donovani* amastigotes in a continuous macrophage-like cell culture. *Journal of Protozoology* **26**, 453–6.

BERENS, R. L., BRUN, R. & KRASSNER, S. M. (1976). A simple monophasic medium for axenic culture of hemoflagellates. *Journal of Parasitology* **62**, 360–5.

BERMAN, J. D., DWYER, D. M. & WYLER, D. J. (1979). Multiplication of *Leishmania* in human macrophages in vitro. *Infection and Immunity* **26**, 375–9.

BERMAN, J. D. & NEVA, F. A. (1981). Effects of temperature on multiplication of *Leishmania* amastigotes within human monocyte-derived macrophages *in vitro*. *American Journal of Tropical Medicine and Hygiene* **30**, 318–21.

BIEGEL, D., TOPPER, G. & RABINOVITCH, M. (1983). *Leishmania mexicana*: temperature sensitivity of isolated amastigotes and of amastigotes infecting macrophages in culture. *Experimental Parasitology* **56**, 289–97.

BRAZIL, R. P. (1978). Isolation of the intracellular stages of *Leishmania mexicana amazonensis* using cellulose column. *Annals of Tropical Medicine and Parasitology* **72**, 579–80.

CHANG, K.-P. (1980*a*). Human cutaneous leishmania in a mouse macrophage cell line: propagation and isolation of intracellular parasites. *Science* **209**, 1240–2.

CHANG, K.-P. (1980*b*). Endocytosis of *Leishmania*-infected macrophages. Fluorometry of pinocytic rate, lysosome–phagosome fusion and intralysosomal pH. In *The Host Invader Interplay* (ed. H. Van Den Bossche), pp. 231–4. Amsterdam: Elsevier.

CHANG, K.-P. & DWYER, D. M. (1976). Multiplication of a human parasite (*Leishmania donovani*) in phagolysosomes of hamster macrophages *in vitro*. *Science* **193**, 678–80.

CHANG, K.-P. & DWYER, D. M. (1978). *Leishmania donovani*. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes. *Journal of Experimental Medicine* **147**, 515–30.

CHILDS, G. E., MCROBERTS, M. J. & FOSTER, K. A. (1976). Partial purification of amastigotes from cutaneous lesions of American leishmaniasis. *Journal of Parasitology* **62**, 676–9.

COOMBS, G. H. (1982). Proteinases of *Leishmania mexicana* and other flagellate protozoa. *Parasitology* **84**, 149–55.

COOMBS, G. H., TETLEY, L., MOSS, V. A. & VICKERMAN, K. (1986). Three-dimensional structure of the leishmania amastigote as revealed by computer-aided reconstruction from serial sections. *Parasitology* **92**, 13–23.

COOMBS, G. H., ROBERTSON, C. D. & MOTTRAM, J. C. (1991). Cysteine proteinases of leishmanias. In *Biochemical Protozoology* (ed. Coombs, G. H. & North, M. J.), pp. 208–20. London: Taylor & Francis.

DOYLE, P. S., ENGEL, J. C., GAM, A. A. & DVORAK, J. A. (1989). *Leishmania mexicana mexicana*: quantitative analysis of the intracellular cycle. *Parasitology* **99**, 311–16.

DOYLE, P. S., ENGEL, J. C., PIMENTA, P. F., DA SILVA, P. P. & DWYER, D. M. (1991). *Leishmania donovani*: long-term culture of axenic amastigotes at 37 °C. *Experimental Parasitology* **73**, 326–34.

EPERON, S. & MCMAHON-PRATT, D. (1989*a*). Extracellular cultivation and morphological characterization of amastigote-like forms of *Leishmania panamensis* and *L. braziliensis*. *Journal of Protozoology* **36**, 502–10.

EPERON, S. & MCMAHON-PRATT, D. (1989*b*). Extracellular amastigote-like forms of *Leishmania panamensis* and *L. braziliensis*. II. Stage- and species-specific monoclonal antibodies. *Journal of Protozoology* **36**, 510–18.

GLASER, T. A., BAATZ, J. E., KREISHMAN, G. P. & MUKKADA, A. J. (1988). pH homeostasis in *Leishmania donovani* amastigotes and promastigotes. *Proceedings of the National Academy of Sciences, USA* **85**, 7602–6.

GLASER, T. A., WELLS, S. J., SPITHILL, T. W., PETTITT, J. M., HUMPHRIS, D. C. & MUKKADA, A. J. (1990). *Leishmania major* and *L. donovani*: a method for rapid purification of amastigotes. *Experimental Parasitology* **71**, 343–5.

HART, D. T., VICKERMAN, K. & COOMBS, G. H. (1981*a*). A quick simple method for purifying *Leishmania mexicana* amastigotes in large numbers. *Parasitology* **82**, 345–55.

HART, D. T., VICKERMAN, K. & COOMBS, G. H. (1981*b*). Transformation *in vitro* of *Leishmania mexicana* amastigotes to promastigotes: nutritional requirements and the effect of drugs. *Parasitology* **83**, 529–41.

HENDRICKS, L. D., WOOD, D. E. & HAJDUK, M. E. (1978). Haemoflagellates: commercially available liquid media for rapid cultivation. *Parasitology* **76**, 309–16.

INFANTE, R. B., HERNANDEZ, A. G., RIGGIONE, F. & DAWIDOWICZ, K. (1980). A new method for the partial purification of leishmania amastigotes from cutaneous lesions. *Parasitology* **80**, 105–12.

LOCKWOOD, B. C., NORTH, M. J., MALLINSON, D. J. & COOMBS, G. H. (1987). Analysis of *Leishmania* proteinases reveals developmental changes in species-specific forms and a common 68-kDa activity. *FEMS Microbiology Letters* **48**, 345–50.

LOOKER, D. L., MARTINEZ, S., HORTON, J. M. & MARR, J. J. (1986). Growth of *Leishmania donovani* amastigotes in the continuous human macrophage cell line U937: studies of drug efficacy and metabolism. *Journal of Infectious Diseases* **154**, 323–7.

MALLINSON, D. J. & COOMBS, G. H. (1986). Molecular characterisation of the metacyclic forms of *Leishmania*. *IRCS Medical Science* **14**, 557–8.

MALLINSON, D. J. & COOMBS, G. H. (1989). Biochemical characteristics of the metacyclic forms of *Leishmania major* and *L. mexicana mexicana*. *Parasitology* **98**, 7–15.

MARR, J. J. (1980). Carbohydrate metabolism in *Leishmania*. In *Biochemistry and Physiology of Protozoa*, Vol. 3 (ed. M. Levandowsky and S. H. Hutner), pp. 313–40. London: Academic Press.

MARTINEZ, S., LOOKER, D. L. & MARR, J. J. (1988). A tissue culture system for the growth of several species of *Leishmania*: growth kinetics and drug sensitivities. *American Journal of Tropical Medicine and Hygiene* **38**, 304–7.

MEADE, J. C., GLASER, T. A., BONVENTRE, P. F. & MUKKADA, A. J. (1984). Enzymes of carbohydrate metabolism in *Leishmania donovani* amastigotes. *Journal of Protozoology* **31**, 156–61.

MUKKADA, A. J., MEADE, J. C., GLASER, T. A. & BONVENTRE, P. F. (1985). Enhanced metabolism of *Leishmania donovani* amastigotes at acid pH: an adaptation for intracellular growth. *Science* **229**, 1099–101.

NORTH, M. J. & COOMBS, G. H. (1981). Proteinases of *Leishmania mexicana* amastigotes and promastigotes: analysis by gel electrophoresis. *Molecular and Biochemical Parasitology* **3**, 293–300.

PAN, A. A. (1984). *Leishmania mexicana*: Serial cultivation of intracellular stages in a cell-free medium. *Experimental Parasitology* **58**, 72–80.

PAN, A. A. & HONIGBERG, B. M. (1985). *Leishmania mexicana pifanoi*: *in vivo* and *in vitro* interactions between amastigotes and macrophages. *Zeitschrift für Parasitenkunde* **71**, 3–13.

PAN, A. A. & MCMAHON-PRATT, D. (1988). Monoclonal antibodies specific for the amastigote stage of *Leishmania pifanoi*. I. Characterization of antigens associated with stage- and species-specific determinants. *Journal of Immunology* **140**, 2406–14.

PAN, A. A., MCMAHON-PRATT, D. & HONIGBERG, B. M. (1984). *Leishmania mexicana pifanoi*: antigenic characterization of promastigote and amastigote stages by solid-phase radioimmunoassay. *Journal of Parasitology* **70**, 834–5.

PAN, A. A. & PAN, S. C. (1986). *Leishmania mexicana*: comparative fine structure of amastigotes and promastigotes *in vitro* and *in vivo*. *Experimental Parasitology* **62**, 254–65.

PUPKIS, M. F. & COOMBS, G. H. (1984). Purification and characterization of proteolytic enzymes of *Leishmania mexicana mexicana* amastigotes and promastigotes. *Journal of General Microbiology* **130**, 2375–83.

PUPKIS, M. F., TETLEY, L. & COOMBS, G. H. (1986). *Leishmania mexicana*: amastigote hydrolases in unusual lysosomes. *Experimental Parasitology* **62**, 29–39.

RAINEY, P. M., SPITHILL, T. W., MCMAHON-PRATT, D. & PAN, A. A. (1991). Biochemical and molecular characterization of *Leishmania pifanoi* amastigotes in continuous axenic culture. *Molecular and Biochemical Parasitology* **49**, 111–18.

RIVAS, L. & CHANG, K.-P. (1983). Intraparasitophorous vacuolar pH of *Leishmania mexicana*-infected macrophages. *Biological Bulletin* **165**, 536–7.

ROBERTSON, C. D. & COOMBS, G. H. (1990). Characterisation of three groups of cysteine proteinases in the amastigotes of *Leishmania mexicana mexicana*. *Molecular and Biochemical Parasitology* **42**, 269–76.

SACKS, D. L., BARRAL, A. & NEVA, F. A. (1983). Thermosensitivity patterns of Old vs. New World cutaneous strains of *Leishmania* growing within mouse peritoneal macrophages *in vitro*. *American Journal of Tropical Medicine and Hygiene* **32**, 300–4.

SARAIVA, E. M. B., PIMENTA, P. F. P., PEREIRA, M. E. A. & DE SOUZA, W. (1983). Isolation and purification of amastigotes of *Leishmania mexicana amazonensis* by a gradient of metrizamide. *Journal of Parasitology* **69**, 627–9.

SCOTT, P. A., JAMES, S. & SHER, A. (1985). The respiratory burst is not required for killing of intracellular and extracellular parasites by a lymphokine-activated macrophage cell line. *European Journal of Immunology*, **15**, 553–8.

SHAPIRA, M., MCEWEN, J. G. & JAFFE, C. L. (1988). Temperature effects on molecular processes which lead to stage differentiation in *Leishmania*. *EMBO Journal* **7**, 2895–901.

SMEJKAL, R. M., WOLFF, R. & OLENICK, J. G. (1988). *Leishmania braziliensis panamensis*: increased infectivity resulting from heat shock. *Experimental Parasitology* **65**, 1–9.

ZILBERSTEIN, D., BLUMENFELD, N., LIVEANU, V., GEPSTEIN, A. & JAFFE, C. L. (1991). Growth at acidic pH induces an amastigote stage-specific protein in *Leishmania* promastigotes. *Molecular and Biochemical Parasitology* **45**, 175–8.