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Characterisation of a *Leishmania mexicana* knockout lacking guanosine diphosphate-mannose pyrophosphorylase

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Abstract

In eukaryotes, the enzyme GDP-mannose pyrophosphorylase (GDP-MP) is essential for the formation of GDP-mannose, the donor of activated mannose for all glycosylation reactions. Unlike other eukaryotes, where deletion of GDP-mannose pyrophosphorylase is lethal, deletion of this gene in *Leishmania mexicana* has no effect on viability, but leads to the generation of avirulent parasites. In this study, we show that the null mutants have a perturbed morphology and cytokinesis, retarded growth and increased adherence to the substratum where they form large colonies. The null mutants attach avidly to mouse macrophages, but unlike the wild type organisms, they do not bind to the complement receptor 3 and are slow to induce phagocytosis. Once internalised, they localise to the phagolysosome, but in contrast to wild type organisms which transform into the intracellular amastigote and establish in the macrophage, they are cleared by 24 h in culture and by 5 h in vivo. The null mutants are hypersensitive to human but not mouse complement and to temperature and acidic pH. Surprisingly, in view of the lack of several known host-protective antigens, injection of the mutant parasites into BALB/c mice confers significant and long lasting protection against infection, suggesting that these temperature sensitive mutants are an attractive candidate for a live attenuated vaccine.

Keywords: *Leishmania*; GDP-mannose pyrophosphorylase; Temperature sensitive mutant; Cytokinesis

1. Introduction

Leishmania are parasitic protozoa responsible for the spectrum of diseases known as leishmaniasis, which range in severity from localised self-limiting cutaneous ulcers to potentially fatal visceral infections (Herwaldt, 1999). Sandflies inoculate motile flagellated promastigotes into the mammalian host in a blood-pool formed during the feeding process (Bogdan et al., 1996; Handman, 1999). Successful infection depends on the parasites' ability to subvert the host innate immune system by binding to receptors on susceptible macrophages followed by phagocytosis and transformation into obligate intracellular amastigotes which resist intracellular destruction. Amastigotes reside in a phagolysosome where they replicate by binary fission, and from which they emerge to reinvade new host cells by receptor-mediated phagocytosis (Chang and Dwyer, 1978; Russell, 1995; Bogdan et al., 1996).

Compared with promastigotes, the amastigotes are much better adapted to intracellular survival, but the mechanisms involved in the transformation of extracellular promastigotes into intracellular amastigotes are still poorly understood. Temperature and pH are important, but not sufficient for this transformation (Teixeira et al., 2002). Transformation of promastigotes into amastigotes is associated with major changes in morphology, gene expression, protein phosphorylation, glucose catabolism, purine metabolism, utilisation of fatty acids, nucleases and cysteine proteases

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(Zilberstein and Shapira, 1994; Bente et al., 2003). A striking aspect of transformation from promastigotes to amastigotes is the dramatic down-regulation of most of the major parasite surface glycoconjugates, with the exception of the glycoinositolphospholipids (GIPLs) which do not change (Descoteaux and Turco, 1999; Ilgoutz and McConville, 2001).

Leishmania promastigotes synthesise a complex glycocalyx that is involved in host–parasite interactions and is important in parasite survival in the sandfly midgut and the macrophage phagolysosome (Beverley and Turco, 1998; Turco et al., 2001). The major cell surface glycoconjugates are lipophosphoglycan (LPG), GIPLs, glycosylphosphatidylinositol (GPI) membrane anchored proteins such as leishmanolysin (gp63) and a family of proteophosphoglycans (PPGs) (Ilgoutz and McConville, 2001).

Mannose is an obligatory sugar in GPI-protein anchors, the GIPLs, the *N*-glycosylated proteins and the phosphoglycan repeats and oligosaccharide caps of LPG and PPGs (Garami and Ilg, 2001a,b; Garami et al., 2001; Ilgoutz and McConville, 2001). Activation of mannose for incorporation into these molecules requires the sequential action of phosphomannose isomerase (PMI), phosphomannomutase (PMM), and GDP-mannose pyrophosphorylase (GDP-MP) to produce GDP-mannose, an essential mannose donor (Burda and Aebi, 1999).

Recently, several genes in the mannose-activation pathway have been deleted experimentally by targeted gene deletion in *Leishmania mexicana* (Garami and Ilg, 2001a,b; Garami et al., 2001). The PMI null mutants (ΔPMI) which lacked LPG, GPI-anchored proteins and GIPLs showed normal morphology, but grew slowly in culture unless provided with mannose, which restored normal growth and glycoconjugate synthesis. They infected macrophages in vitro as well as wild type parasites and showed only a slower rate of lesion progression when injected into mice. This normal phenotype may be due to the fact that the ΔPMI can scavenge mannose from the culture medium in vitro and from tissue fluid in the infected host. In contrast, the ΔPMM and the ΔGDP-MP parasites, which lacked all mannose-containing glycoconjugates, although viable in culture, were avirulent and were unable to survive in macrophages in vitro or mice in vivo infection.

The successful targeted deletion of the *GDP-MP* gene in *Leishmania* was surprising because in yeast GDP-MP is essential for survival and homozygous targeted gene deletion is lethal. When GDP-MP expression was down regulated (but not totally deleted) in *Saccharomyces cerevisiae* and *Candida albicans*, the major phenotypes observed were cell lysis, rounded swollen cells, failure of daughter-cell separation and/or cytokinesis, impaired bud growth as well as clumping and flocculation in culture (Warit et al., 2000). A similar phenotype was observed in a mutant of *Hansenula polymorpha* that displayed reduced GDP-MP activity (Agaphonov et al., 2001). No naturally occurring gene deletion or mutations of *GDP-MP* have been described in mammals, suggesting that it is essential for life.

In this study, we investigated the cell biology of the null mutant ΔGDP-MP parasites and their interaction with their host cells, with a view to elucidate the mechanisms contributing to their avirulent phenotype. We show here that the ΔGDP-MP parasites have a disturbed morphology, cytokinesis and growth, and are extremely sensitive to acidic pH, temperature and human, but not mouse complement. ΔGDP-MP are able to invade macrophages but the kinetics of uptake are slow. In the macrophage, the mutants localise normally to the phagolysosome, but are cleared rapidly both in vitro and in vivo.

The ΔGDP-MP are the first example of a temperature sensitive avirulent *Leishmania* mutant. We show that they are an attractive candidate for a live attenuated *Leishmania* vaccine.

2. Materials and methods

2.1. Parasites

The GDP-mannose pyrophosphorylase null mutant ΔGDPMP:YG ΔGDPMP:BLE (ΔGDP-MP) is a cloned line generated by targeted gene deletion derived from *L. mexicana* MNYC/BZ/62/M379 (Garami and Ilg, 2001b). ΔGDP-MP and wild type MNYC/BZ/62/M379 (M379) promastigotes were maintained in vitro at 26 °C in pH 7.5 semi-defined medium 79 (SDM) (Gibco BRL) supplemented with 10% (v/v) heat-inactivated FCS (Trace Biosciences) and 2 mM L-glutamine (BDH Laboratory Supplies). In some experiments cultures were grown at 33 °C or in medium where the pH was adjusted to pH 5.5 with hydrochloric acid.

2.2. Parasite growth kinetics

Promastigotes were harvested from stationary phase cultures and diluted to 1×10^5 ml^{-1} in 10 ml of medium. At 24 h intervals the parasite concentration and viability were determined microscopically following dye exclusion staining with 2.5 μg/ml ethidium bromide (Sigma Chemical Co.) and 2.5 μg/ml acridine orange in phosphate buffered saline (PBS) (Aldrich Chemical Co.). In some experiments wild type M379 and ΔGDP-MP growth kinetics were compared under conditions of heat or acid stress. Cultures were grown under four different conditions, namely, normal promastigote culture (26 °C, pH 7.5), acidified medium (26 °C, pH 5.5), increased temperature (33 °C, pH 7.5) and a combination of 33 °C and pH 5.5, previously established for the generation of axenic amastigotes (Pan et al., 1993). For these experiments, 2.5×10^6 stationary phase promastigotes were inoculated into 5 ml of medium.

2.3. Morphological examination of the ΔGDP-MP parasites

Wild type or ΔGDP-MP promastigotes that had been cultured for 24 h at either 26 °C pH 7.5, 26 °C pH 5.5, 33 °C pH 7.5 or 33 °C pH 5.5 were harvested, washed with PBS and fixed in suspension in 4% paraformaldehyde in PBS. The parasite suspensions were allowed to adhere onto glass microscope slides coated with poly-L-lysine (Sigma) and stained using the DiffQuick kit (LabAids). Images were taken under oil immersion using a Nikon Coolpix 995 camera (Nikon) and an Eclipse ∈600 microscope (Nikon). A Diaphot 300 inverted microscope (Nikon) and KX series imaging system (Apogee) were used to record images of low and high density ΔGDP-MP cultures growing in flasks at 26 °C pH 7.5.

2.4. Mice

Female BALB/c mice aged 6–8 weeks were obtained from the Walter and Eliza Hall Institute specific pathogen-free breeding facility and maintained under conventional conditions. The studies are in line with the NHMRC Code of Practice for the Care and Use of Animals for Scientific Purposes and have been approved by the Intercampus Animal Ethics Committee (AEC #2002.20).

2.5. In vivo infections

The in vivo virulence of the ΔGDP-MP parasites was determined by intradermal injection of 1×10^7 ΔGDP-MP promastigotes in stationary phase of growth. Subsequent lesion development was monitored using a semi-quantitative scoring system based on the diameter of the lesion (Mitchell, 1983). Short-term survival and long-term persistence of the ΔGDP-MP parasites in vivo were investigated by culturing the skin excised from the site of infection and the cells from draining lymph nodes, spleen, kidney, subcutaneous tissue and non-draining lymph nodes, 1, 5, 24 h, or 2 months p.i.

2.6. Vaccination experiments

In two independent experiments groups of six or 12 BALB/c mice were vaccinated by i.p. or s.c. injection of 2×10^7 ΔGDP-MP stationary phase live or promastigotes killed by fixation in 1% paraformaldehyde 2% glucose in PBS followed by a booster injection of 2×10^7 promastigotes of the same type 2 weeks later. The control mice were injected with PBS.

Three weeks post-vaccination, all mice were challenged by intradermal injection of 1×10^7 stationary phase virulent wild type M379 promastigotes at the base of the tail. Lesion development was monitored as described above. Twelve weeks or 20 weeks p.i. two or three mice from each group were killed and draining lymph nodes were collected and the parasite burden was determined by limiting dilution analysis (Titus et al., 1985).

The average weekly change in the lesion size was calculated for each mouse and a statistical permutation test which is part of the 'statmod' package for R was used to compare the vaccinated and control mice (Team, R.D.C., 2004. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). The *P* values were adjusted using a step-down Bonferroni method for multiple testing (Holm, 1979).

2.7. In vitro infection of macrophages

The macrophage cell line J774 (ATCC) or resident peritoneal macrophages were used to examine the ability of the mutant parasites to invade and survive. Resident peritoneal macrophages were harvested from 6 to 8-week-old female BALB/c mice by peritoneal lavage. Cells were washed and resuspended in DME medium (Gibco BRL) supplemented with 10% FCS. 5×10^4 cells were incubated in four-well plates on 13 mm sterile glass coverslips (Menzel-glaser) at 37 °C. Non-adherent cells were washed away after overnight incubation and before infection.

All in vitro infections were performed with parasites in the stationary phase of growth. Macrophages were infected at 33 °C with either a 2:1 or 5:1 parasite to macrophage multiplicity of infection (MOI). After 5 h, free parasites were removed by washing, and the cells were either fixed and stained immediately with Giemsa (manufacturer), or cultured for an additional 24 h before staining.

Several *Leishmania* surface glycoconjugates such as gp63 and LPG that are absent in the ΔGDP-MP parasites have been implicated in the parasite invasion through binding to macrophage CR3, a receptor that does not trigger the oxidative burst in macrophages (Bogdan et al., 2000; Stafford et al., 2002). In view of the difference in the phagocytosis and survival of the mutants in macrophages, we examined whether the ΔGDP-MP parasites bind to CR3 and use this pathway to gain entry into the macrophage. Two anti-CR3 monoclonal antibodies were used to inhibit infection with wild type or mutant promastigotes, 5C6 and M1/70 (ATCC) directed against the CD11β chain of CR3. In two independent experiments done in duplicate, a control antibody directed against the *Plasmodium falciparum* SERA5 protein (a generous gift from Anthony Hodder) or normal rat IgG (Chemicon International) were used. For these experiments, the cells were incubated with 15 μg of antibody for 45 min before infection as described above. After 45 min incubation, free parasites were removed by washing and the cells incubated for a further 90 min.

For infection with opsonised parasites, promastigotes were incubated for 30 min with 4% C5-deficient mouse serum in medium before addition to the macrophage monolayer and the level of infection and survival were quantitated after 5 or 24 h as above (Racoosin and Beverley,

1997). The effect of antibodies to the CR3 was also examined by incubating the macrophages with M1/70 before the addition of the opsonised parasites as above, except that the infection was allowed to proceed for 5 or 24 h. In all experiments, infection was determined by microscopic examination of duplicate samples stained with 5% Giemsa or the DiffQuick staining kit (manufacturer). Cells (400 or 500) were counted on each of duplicate coverslips.

2.8. Western blot analysis

Cell lysates prepared from 1×10^6 promastigotes in Laemmli SDS-sample buffer containing 50 mM DTT (BioRad) were incubated for 5 min at 90 °C and the proteins separated by electrophoresis on 10% polyacrylamide gels (Laemmli, 1970). Western blotting was performed as described previously (Towbin et al., 1979) using Protran nitrocellulose membrane (Schleicher and Schuell). The expression of GDP-MP was detected with rabbit antibodies to the recombinant DNA-derived *L. mexicana* GDP-MP followed by horseradish peroxidase-conjugated sheep anti-rabbit IgG (Silenus Laboratories). Binding was detected using the enhanced chemiluminescence (ECL) detection system (Amersham).

2.9. Transmission electron microscopy

A semi-simultaneous fixation protocol (Tippit and Pickett-Heaps, 1977) was used to prepare samples for TEM. Briefly, cells were fixed for 5–10 s with 0.5% glutaraldehyde (ProSciTech) in PBS followed by addition of an equal volume of 1% OsO_4 (ProSciTech) in PBS for 20 min. The samples were washed three times in distilled H_2O then stained overnight at 4 °C with 2% aqueous uranyl acetate (ProSciTech) followed by dehydration in a graded series of methanol from 5 to 100%. Following dehydration, samples were infiltrated with increasing concentrations of LR White resin up to 100% (ProSciTech) and polymerised at 70 °C overnight.

Sections prepared using a Leica Ultracut R ultramicrotome (Leica Microsystems) were collected on pioloform-coated (Alltech) copper grids (ProSciTech) and post-stained in 2% aqueous uranyl acetate (ProSciTech) and a triple-lead solution. The sections were examined on a Philips BioTwin CM 120 transmission electron microscope, and micrographs taken on Kodak electron microscope film 4489 (Eastman Kodak Co.).

2.10. Immunofluorescence microscopy

Macrophages cultured on coverslips and infected with either wild type M379 or ΔGDP-MP parasites were examined by immunofluorescence microscopy. The samples were fixed with 4% paraformaldehyde, followed by quenching of all reactive sites with 50 mM NH_4Cl, then 10% FCS in PBS. The cells were permeabilised in 0.1 mg ml^{-1} saponin and incubated for 1 h at room temperature with antibodies to the transferrin receptor (TfR), macrosialin, lysosomal-associated membrane protein 1 (LAMP-1) (PharMingen), Cathepsin D (CatD) and Cathepsin B (CatB) (a kind gift from J.C. Antoine, Pasteur Institute, Paris). Binding was detected with FITC-conjugated sheep anti-rabbit or mouse IgG (Silenus laboratories). Coverslips were mounted in Mowiol mounting medium (Calbiochem) and examined by confocal fluorescence microscopy using a Leica DMIRE2 microscope (Leitz) and a Leica TCS SP2 imaging system (Leitz).

2.11. Mmc microscopy

J774 macrophages cultured on coverslips were mounted in a perfusion chamber similar to that described by McGee and Allen (1971) and sealed with VALAP (vaseline: lanolin: paraffin 1:1:1 by weight). The perfusion chamber was filled with culture medium and connected to two reservoirs (plastic syringes) through a two-way stopcock. The perfusion chamber allowed exchange of culture medium, admittance of either wild type or ΔGDP-MP promastigotes and the exchange of experimental medium (26 °C pH 7.5, 26 °C pH 5.5, 33 °C pH 7.5 or 33 °C pH 5.5) to the chamber while maintaining focus during recording. Promastigotes were used in early or late logarithmic phase and observed over a period of 1–4 h. The temperature of the microscope stage was kept at room temperature for the promastigote studies or adjusted to 30 °C for the macrophage infection studies by means of an air curtain. The temperature was monitored on the stage with a temperature sensor.

Images were recorded using a F250-CCD colour mmc camera (Panasonic) mounted to a Leica DMIRB inverted microscope (Leitz) and observed with a phase 40×/1.00 NA oil or a phase 100×/1.30 NA oil immersion objective (Plan-Fluotar) using phase contrast or differential interference contrast (DIC) optics. Images were captured in real time on a high-density metal particle tape (MII) using a MII AU-650B Video Cassette Recorder (Panasonic) and later transcribed to time-lapse onto a VDR-V1000P Rewritable videodisc recorder (Pioneer).

2.12. Human complement lysis assay

The susceptibility of the ΔGDP-MP parasites to lysis by human complement was compared to that of wild type organisms in an in vitro killing assay (Späth et al., 2003). One million stationary phase promastigotes in 200 µl SDM were mixed with an equal volume of medium supplemented with graded concentrations of fresh normal human serum and incubated at room temperature for 30 min. The parasites were then pelleted by centrifugation at 900 g and resuspended in 200 µl ice cold PBS. Viability counts were

performed after staining with acridine orange and ethidium bromide as described above.

3. Results

3.1. Leishmania mexicana ΔGDP-MP promastigote morphology and cytokinesis are perturbed

In this study, we have expanded the initial biochemical characterisation of the ΔGDP-MP organisms, by examining the effect of the loss of all mannose-containing molecules on the parasite cell biology and on the molecular interactions with the host macrophage.

The most striking observation of cultures of the ΔGDP-MP promastigotes is their increased adherence to plastic culture flasks and to each other (Fig. 1 and Supplementary data). From the very early time points in logarithmic phase the parasites attach to the plastic dish, and as they start to divide, the daughter cells remain attached to each other (Fig. 1A). This is in contrast to the wild type promastigotes, which grow as individuals or in small rosettes that separate in the late logarithmic phase of growth and do not attach to plastic (Supplementary data). This behaviour prevented the capture of the wild type organisms on film. Immunofluorescence staining with antibodies to α tubulin confirmed that in these cultures a significant number of organisms the daughter cells had not separated (data not shown). With time in culture, they form firmly attached large colonies (Fig. 1B).

In contrast to the wild type, the ΔGDP-MP promastigotes display a heterogeneous morphology, with 30–50% normal elongated organisms, but 40–60% showing a body half the length of wild type organisms and 10–20% rounded very small forms (Supplementary data). The length of the flagella is also heterogeneous, some have no flagella and many appear severely truncated (Supplementary data). The ΔGDP-MP promastigotes move more sluggishly than the wild type parasites and the flagella often remain attached to the substratum where they anchor the promastigotes (Supplementary data).

Treatment of the cell culture with 0.5 mg ml^{-1} trypsin and 5 mM EDTA in PBS dispersed the parasite clumps, but EDTA alone had no effect, suggesting that proteins are involved in the adhesive phenotype of the mutant promastigotes, but that these interactions are not dependent on the presence of divalent cations (data not shown).

3.2. Growth kinetics of the ΔGDP-MP promastigotes

Our studies confirmed the data described in the original manuscript of Garami and Ilg, showing that under the culture conditions of 26 °C and pH 7.5 used routinely for the *Leishmania* promastigotes, the ΔGDP-MP parasites showed retarded growth compared with wild type parasites (Fig. 2A). Our data suggest that these effects could be explained by a combination of a lower rate of cell division, or daughter-cell separation during the early logarithmic growth phase, and a lower population density at which stationary phase is reached and parasites start to die (Fig. 2A). The wild type promastigotes reached a peak on day 5 and their numbers decreased gradually by about 30% over the next 4 days. The mutant promastigotes reached a peak at a much lower density on day 7, but the number of live promastigotes decreased by 50% over the next 2 days (Fig. 2A).

3.3. In wild type promastigotes, GDP-MP expression is constant during the logarithmic and stationary phase of growth

Promastigote development in culture is thought to mimic the maturation of the parasites in the sandfly vector. Thus, the less virulent procyclic promastigotes are present in the logarithmic phase of growth and highly virulent metacyclic parasites are detected in late stationary phase. A major feature of metacyclogenesis is the remodelling of the promastigote surface glycocalyx as well as the synthesis of other mannose-containing glycans (Sacks, 1989; Sacks et al., 1990; Ralton et al., 2003). We set out to investigate whether an increased need to synthesise mannose-containing glycoconjugates in stationary phase might be associated with an increased

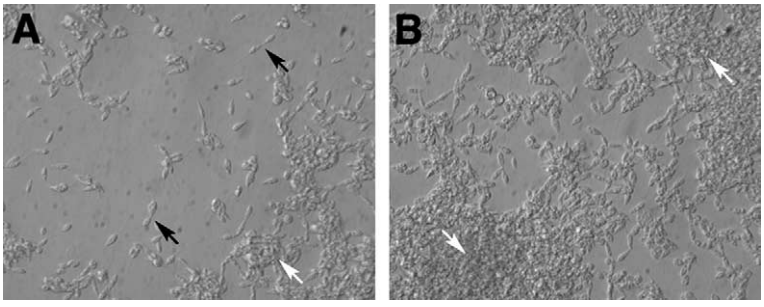

Fig. 1. The adherent phenotype of the ΔGDPMP parasites in culture. DIC light microscopy images of ΔGDP-MP promastigote cultures in flasks at a magnification of 400× (A) early logarithmic phase of growth and (B) late logarithmic phase of growth. Failure of parasites to separate following mitosis can be observed (black arrows). Small ΔGDP-MP colonies increasing in size with time in culture can be observed (white arrows in A and B).

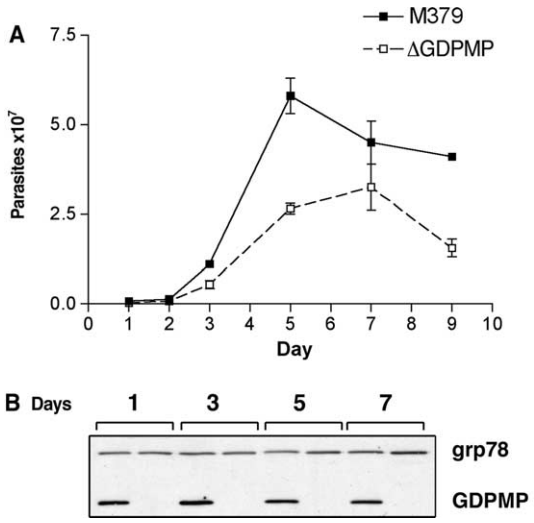

Fig. 2. Wild type M379 and ΔGDP-MP promastigote growth curves and GDP-MP expression. Cultures were initiated with 1×10^5 promastigotes ml^{-1} and counts made at 24 or 48 h intervals (A). The data represent the mean and standard deviation from two independent experiments. Parallel cultures to those used for panel A were used to examine the expression of GDP-MP in wild type and mutant parasites by Western blotting (B). Detergent lysates of 1×10^6 parasites per lane were separated by SDS-PAGE and GDP-MP was detected with rabbit antibodies to the recombinant DNA-derived protein. As a loading control, the glucose regulated protein grp78 was detected on the same blots with antibodies to recombinant grp78 (Jensen et al., 2001). The numbers above each bracket represent the day in culture. Each bracket encompasses two lanes, the left lane containing the M379 wild type parasite lysate and the right lane the ΔGDP-MP parasite lysate.

abundance of GDP-MP in wild type organisms. Fig. 2B shows that the level of expression of GDP-MP in wild type promastigotes is similar over the 7 days of culture. As expected, there was no expression of the enzyme in the ΔGDP-MP parasites (Fig. 2B).

ΔGDP-MP promastigotes attach to macrophages but are slow to be phagocytosed. It was of interest to investigate the interaction between the ΔGDP-MP promastigotes and the macrophage because of the absence of the parasite molecules such as LPG, gp63 and PSA-2, which had been implicated in attachment to macrophages and phagocytosis. Using mmc microscopy, the parasite attachment and phagocytosis process of wild type and null mutants was monitored over several hours (Supplementary data). The ΔGDP-MP promastigotes bound avidly to macrophages, but following attachment they were phagocytosed considerably more slowly than the wild type parasites (Supplementary data). Of 15 interactions of wild type parasites with macrophages observed, the average time taken for internalisation was 4 min 1 s with a standard deviation of 2 min 35 s. In contrast, the average time taken for the mutant parasites during nine interactions with macrophages was 14 min 28 s with a standard deviation of 10 min 15 s. In contrast to the wild type, the binding of the mutants to macrophages did not induce the very active movement of the macrophage membrane. Much less active ruffling and pseudopod formation was observed compared with wild type parasites.

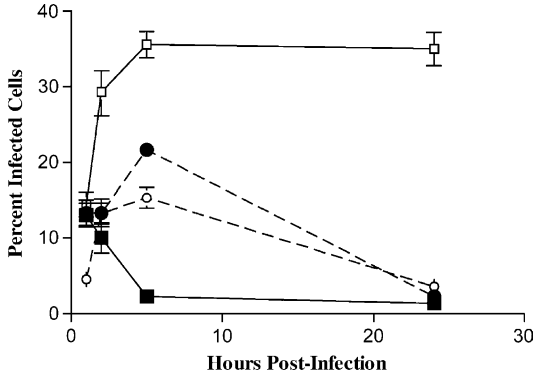

Fig. 3. In vitro infection of peritoneal macrophages with wild type *Leishmania mexicana* M379 or ΔGDP-MP promastigotes. The data represent the mean and standard deviation of 400 cells in each of duplicate samples from two independent experiments. Filled squares indicate percent macrophages with wild type *L. mexicana* attached but not internalised, while blank squares represent macrophages with internalised wild type parasites. Filled circles indicate percent macrophages with attached ΔGDP-MP promastigotes, and blank circles macrophages with internalised ΔGDP-MP.

Inefficient parasite uptake was also demonstrated in experiments in which the number of attached and internalised parasites was quantitated (Fig. 3). In the case of wild type organisms, nearly all of the initially attached parasites were internalised over the first 5 h with about 40% of macrophages infected (Fig. 3). In contrast, only about 15% of the macrophages contained intracellular ΔGDP-MP parasites at 5 h, with over 20% showing attached but not internalised parasites ($P<0.0001$). The video clips examining the interaction of the parasites with the macrophages show that 69% of wild type parasites which had come into contact with the macrophages were internalised, while only 28% of the mutants which had attached were internalised over the same period (Supplementary data). The clusters of mutants, containing 18 ± 16 parasites attached to macrophages but were never seen to be phagocytosed (Supplementary data). In contrast to the wild type organisms, most ΔGDP-MP parasites, which had been internalised, disappeared from the infected cultures by 24 h ($P<0.0001$). The clearance of the mutant parasites at 24 h rather than the 48 h described initially by Garami and Ilg (2001b) is probably due to experimental variability and to differences in the experimental protocol used. The killing of the mutant organisms was even faster in vivo than in vitro. Mutant parasites could only be isolated from the skin 1 h after intradermal injection into BALB/c mice, and no mutant parasites could be isolated from any organ after 5 or 24 h.

3.4. Antibodies to CR3 block infection by wild type promastigotes, but have no effect on ΔGDP-MP

The antibodies 5C6 and M1/70 directed to the β chain of CR3 reduced the wild type parasite infection levels by about 50%, but they had no effect on the level of ΔGDP-MP parasite infection (Fig. 4A, $P<0.001$ for both 5C6 and M1/

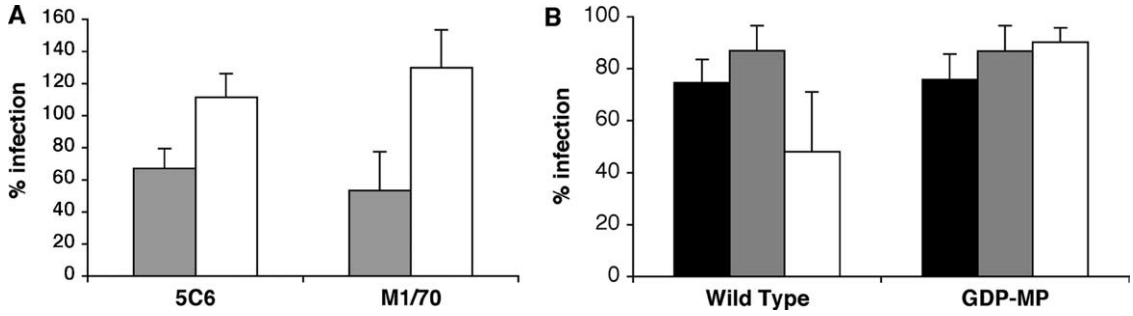

Fig. 4. In vitro infections of macrophages with wild type M379 or ΔGDP-MP in the presence of antibodies to CR3 or C5-deficient serum. Macrophages were infected at a multiplicity of infection of two parasites per cell in the presence of anti-CR3 antibodies 5C6 or M1/70, or a control antibody and the percent inhibition of binding was calculated in relation to controls (A). Filled columns indicate wild type promastigotes and blank columns indicate ΔGDP-MP. The data represent the mean and standard deviation of the normalised percent infection of treated samples compared to untreated controls from two independent experiments performed as in Fig. 3 Cells were also infected with promastigotes opsonised by prior incubation in 4% C5-defficient serum in medium, and infection was quantitated after 5 h in the presence or absence of the M1/70 antibody by counting the total number of parasites associated or internalised by the cells (B) Black columns represent infected macrophages with the untreated control parasites, grey columns show the percent macrophages infected with opsonised parasites, and blank columns represent the percent infected cells in the presence of M1/70.

70). This indicates that CR3 plays no role in the interaction of the mutants with macrophages and other host molecules may mediate invasion in this in vitro system, in the absence of complement. The use of an alternative pathway for phagocytosis may account for the lower efficiency of parasite internalisation despite avid attachment. However, opsonisation of the promastigotes with C5-deficient serum had no significant effect on the initial attachment or phagocytosis of the mutants, and only a marginal effect on the wild type parasites (Fig. 4B). It also did not affect survival of the mutants over the 24 h of observation (data not shown). The monoclonal antibody M1/70 was able to reduce infection by the wild type parasites, but did not seem to affect the mutants (Fig. 4B). The data suggests, but does not prove, that the mutants may not bind mouse complement or that the binding is at a lower affinity and more longer incubations may be necessary.

3.5. The ΔGDP-MP parasites localise to the phagolysosome

Upon phagocytosis, the ΔGDP-MP parasites localise to the same late endosomal lysosomal compartment as the wild type organisms, as demonstrated by the presence of the markers macrosialin, LAMP-1, CatD and CatB in the parasite-containing vacuole within 2 h of phagocytosis (data not shown). However, despite the similar localisation, the in vitro infection studies described in Fig. 3, as well as previously published data (Garami and Ilg, 2001b), indicate that in contrast to the wild type organisms, the ΔGDP-MP parasites are killed following phagocytosis.

3.6. ΔGDP-MP parasites show increased sensitivity to complement-mediated lysis

The loss of several glycoconjugates from the surface glycocalyx has been shown to increase promastigote sensitivity to lysis by human complement (Späth et al., 2003). The ΔGDP-MP parasites are more sensitive than wild type parasites to complement mediated lysis (Fig. 5). At a concentration of 2% normal human serum about 80% of mutant promastigotes were killed, whereas only about 40% of wild type promastigotes were killed, while at a 10% serum concentration all the ΔGDP-MP were killed, but 9% of wild type organisms survived (Fig. 5). As can be seen in Fig. 5 the degree of killing caused by the serum was variable, making the difference between the groups not statistically significant. The heterogeneity may be due to the mixed population of wild type organisms containing procyclic parasites which are susceptible and metacyclic parasites which are resistant. It was necessary to use this heterogeneous population of parasites on day 4 in culture in order to ascertain the viability and health of the mutant parasites (see below).

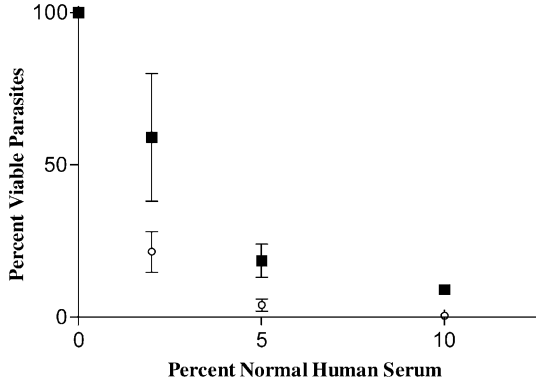

Fig. 5. ΔGDP-MP parasites are sensitive to human complement-mediated lysis. Wild type *Leishmania mexicana* (black squares) and ΔGDP-MP (clear circles) promastigotes harvested on day 4 of culture were incubated for 30 min in medium supplemented with the indicated concentration of fresh normal human serum as a source of complement. The percent live parasites was calculated with respect to untreated controls. The data represent the mean and standard deviation from two independent experiments.

3.7. The ΔGDP-MP parasites are sensitive to temperature and acid pH

The axenic *L. mexicana* amastigotes obtained by growing promastigotes in vitro for several days at 33 °C and the acidic pH 5.5 have been shown to mimic lesion-derived amastigotes in their metabolic profile and their increased virulence and ability to survive in macrophages.

In view of the inability of the mutant promastigotes to survive after infection of macrophages and to produce amastigotes, it was of interest to examine their intrinsic ability to differentiate into amastigotes in a host cell-free system. We also wondered if such differentiation was possible and if so, once amastigotes were produced, whether they would be able to infect macrophages and survive intracellularly.

Wild type promastigotes incubated for 24 h at 33 °C and pH 5.5 assumed a more stumpy shape, and some had lost their flagella and acquired a morphology similar to amastigotes (Fig. 6A). Transformation of wild type parasites into amastigotes under these conditions was not complete until after about 72 h. In contrast, ΔGDP-MP parasites had already rounded up by 24 h, lost their flagella, clumped and displayed multiple large vacuoles (Fig. 6A). In marked contrast to the wild type parasites, which by 48 h showed significant numbers of replicating amastigotes, most mutant parasites were dead after 48 h at 33 °C (data not shown).

In order to dissect out the contribution of the acid pH and that of the increased temperature to the death of the mutants, we cultured the parasites either in acidic medium at 26 °C or in normal medium at 33 °C. At pH 5.5 and 26 °C the wild type parasites retained a highly motile promastigote morphology (Fig. 6A). In contrast, the ΔGDP-MP parasites assumed a rounded morphology with large vacuoles throughout the cytoplasm and formed extremely dense disorganised clumps. (Fig. 6A). After 24 h in culture at pH

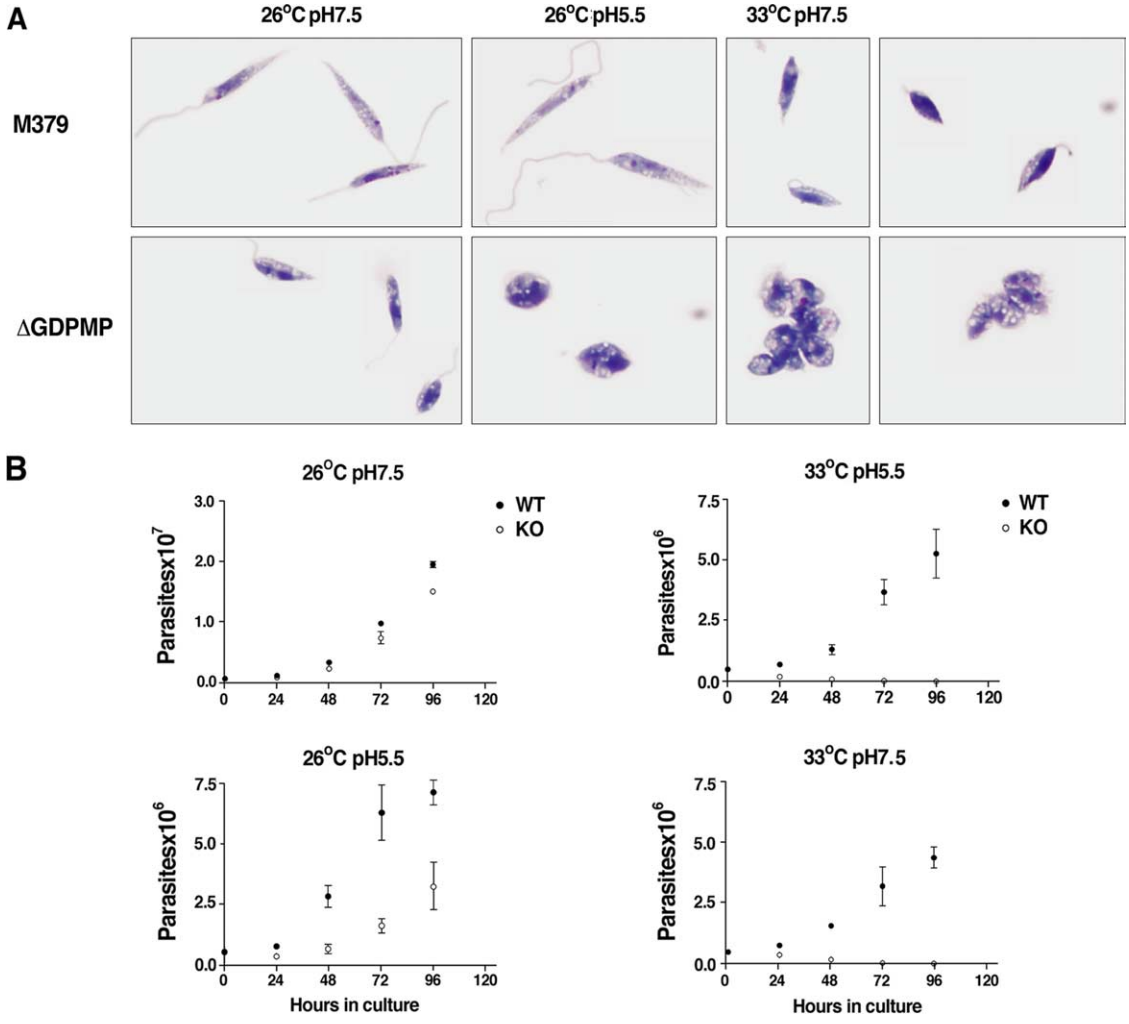

Fig. 6. Morphology and survival of M379 and ΔGDP-MP parasites in response to stress induced by heat and acid pH. The morphology of Giemsa stained M379 and ΔGDP-MP parasites was recorded using light microscopy after 24 h incubation under the indicated culture conditions of either 26 °C pH 7.5 or 26 °C pH 5.5, or 33 °C pH 7.5 or 33 °C pH 5.5 (A). All images were taken under oil immersion at a final magnification of 1000×. The growth kinetics of the parasites cultured under the conditions described in (A) were determined over 4 days (B). The data represent the mean and standard deviation from two independent experiments.

7.5, but at the increased temperature of 33 °C, the wild type population was heterogeneous with mostly stumpy forms and a few amastigotes (Fig. 6A). This was in agreement with earlier studies that have shown that temperature alone can trigger a degree of transformation (Bates, 1993). Under these conditions, the ΔGDP-MP parasites assumed an amastigote-like morphology, but their entire cytoplasm became filled with vacuoles (Fig. 6A). Dense aggregates of ΔGDP-MP parasites formed, and these often appeared to contain large amounts of cellular debris from dead cells.

When the mutant parasites were grown either at 33 °C and pH 5.5 or at 33 °C and pH 7.5, they died over a 48 h period (Fig. 6B). Parasites exposed to a combination of high temperature and low pH died more rapidly than those exposed to high temperature alone (Fig. 6B). In contrast to the effect of temperature, which was lethal at both acid and neutral pH, the acid pH alone reduced the rate of parasite growth but did not seem to affect viability (Fig. 6B). Under all the above conditions, the viability of the wild type parasites was not affected (Fig. 6B), but both growth conditions led to slower growth and a 30–50% reduction in parasite numbers.

TEM confirmed the light microscopy observations and showed the appearance of large vacuoles in the mutant parasites grown at acid pH (Fig. 7B), and a total loss of cellular organisation in the null mutants grown at 33 °C and pH 7.5 (Fig. 7D). In contrast, the wild type promastigotes grown at acid pH maintained their morphology (Fig. 7A), but at 33 °C and pH 7.5 they started the transformation into amastigotes with the appearance of the amastigote-specific organelles, the megasomes (Fig. 7C).

3.8. Vaccinations with ΔGDP-MP parasites induces partial protection in mice

The lesions were slow to develop in both vaccinated and control mice and no overt pathology was detected in any of the groups for the first 4 weeks. Despite the variability between individual mice in each group, differences in the disease phenotype became apparent after this time (Fig. 8). In two independent experiments, i.p. vaccinations with live parasites conferred significantly greater protection compared with the s.c. vaccination or control infection (Fig. 8A and C). In the experiment presented in Fig. 8C, the i.p. vaccinated mice showed no lesions during the entire

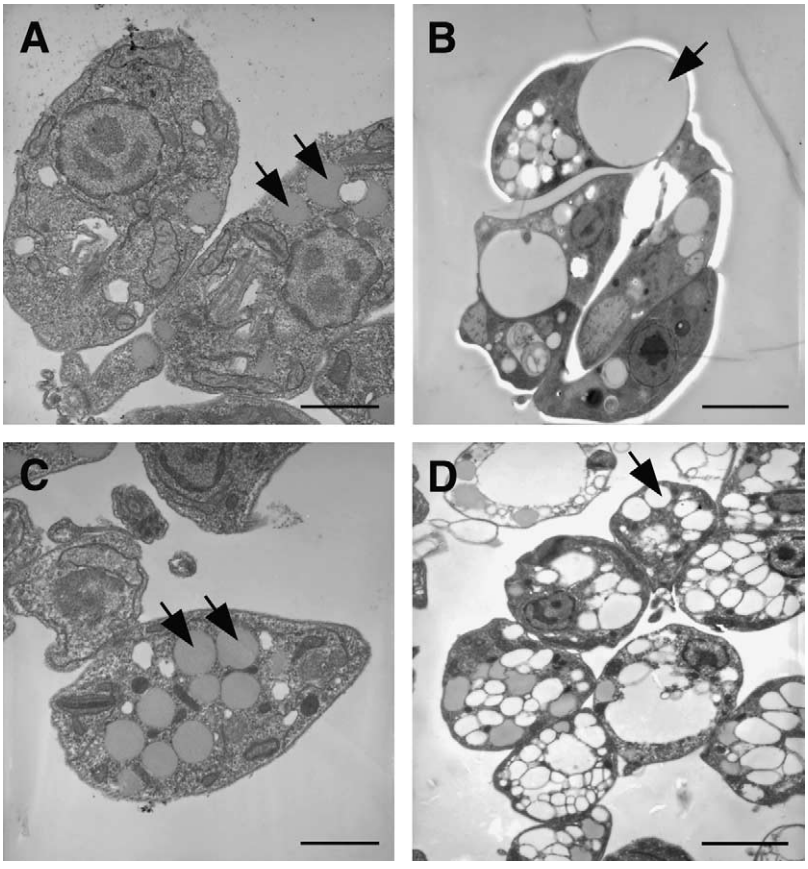

Fig. 7. Structural examination by transmission electron microscopy of M379 and ΔGDP-MP parasites cultured at 33 °C and pH 7.5 or 26 °C and pH 5.5. Wild type (A and C) or ΔGDP-MP (B and D) promastigotes from the cultures described in Fig. 6A were prepared for electron microscopy as described in Section 2. Panel A, wild type promastigotes grown at 26 °C and pH 5.5; Panel C, wild type promastigotes grown at 33 °C and pH 7.5; Panel B, ΔGDP-MP parasites at 26 °C and pH 5.5; Panel D shows mutant parasites grown at 33 °C and pH 7.5. Arrows point to large vacuoles in B and D, and to megasomes in A and C. Magnification is indicated by the 2 μm scale bars.

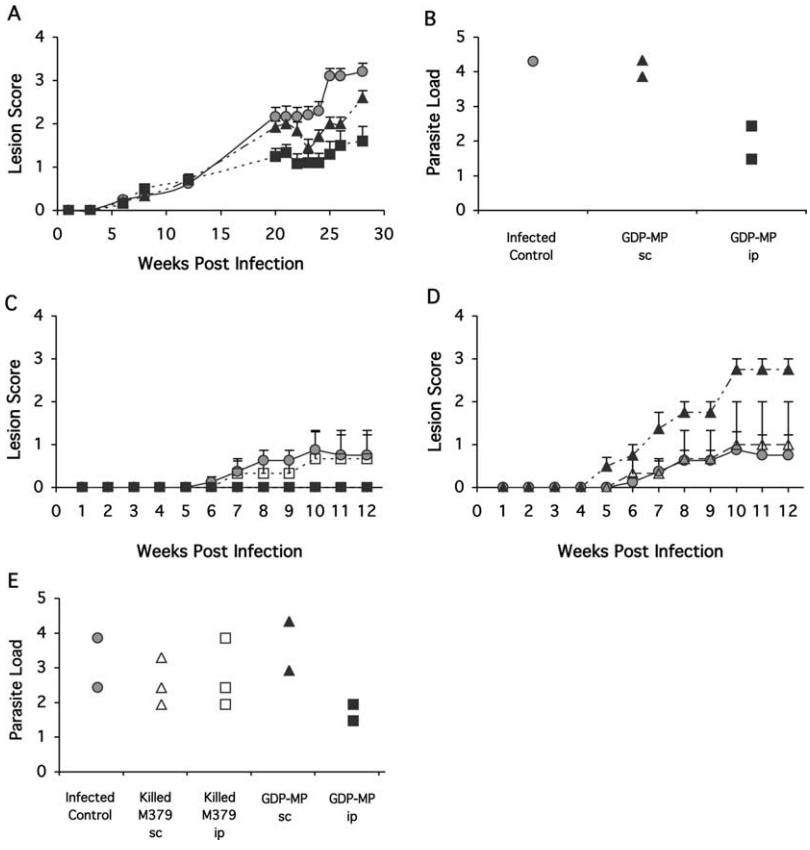

Fig. 8. The pattern of lesion development and parasite burdens following vaccination of BALB/c mice with live ΔGDP-MP or killed wild type promastigotes. Mice were vaccinated by intraperitoneal or subcutaneous injection and challenged by intradermal infection with wild type parasites. The lesion development was monitored weekly (A, C and D). In panels A and B, representing one experiment, the mice were vaccinated intraperitoneally (black squares) or subcutaneously (black triangles) with live ΔGDP-MP or injected with PBS (shaded circles). In a second experiment depicted in panel C the mice were vaccinated intraperitoneally with killed wild type parasites (blank squares) or live ΔGDP-MP (black squares) or PBS (shaded circles), while in panel D they were vaccinated subcutaneolsy with killed wild type (blank triangles) or live ΔGDP-MP parasites (black triangles). The $\log_{10}$ parasite burden per 1×10^6 cells from the draining lymph nodes of two or three mice from each group 12 (E) or 20 (B) weeks after challenge was determined using a limiting dilution analysis as described in Section 2.

12 weeks of observation ($P=0.057$), while in that presented in Fig. 8A, the i.p. vaccinated mice showed significantly smaller lesions than the controls, in particular during the weeks 20–28 ($P=0.0009$). In contrast, mice vaccinated s.c. were no different from the controls (Fig. 8A). In one experiment, mice injected with the knock out parasites s.c. had larger lesions than the control mice (Fig. 8D), but in the other experiments using this vaccination regimen the disease severity was similar to that of the controls. Vaccination with killed parasites via the peritoneal or subcutaneous route afforded no protection (Fig. 8C and D). The mice vaccinated i.p. showed about 100 fold lower parasite burdens compared with the other groups including the mice vaccinated s.c. (Fig. 8B and E).

4. Discussion

The *Leishmania* surface glycocalyx, consisting of GIPLs, LPG, GPI-anchored proteins and *N*-glycosylated proteins has been considered critical for maintaining parasite virulence (Ilgoutz and McConville, 2001). Surprisingly, a series of gene knockouts selectively eliminating several of these surface glycoconjugates had little impact on parasite virulence (Ilg et al., 2000; Garami and Ilg, 2001a). However, the deletion of phosphomannomutase or the GDP-mannose pyrophosphorylase, which led to the global loss of mannose containing glycoconjugates, led to an inability to survive in macrophages or mice (Garami and Ilg, 2001a,b; Garami et al., 2001). These data make the mannose biosynthetic pathway an attractive target for drug development (Davis et al., 2004).

Mannose-containing glycoconjugates are involved in many different functions, so it is hardly surprising that deletion of GDP-MP is found to have pleiotropic effects. Nonetheless, our studies allow these effects to be grouped into those affecting cell morphology and movement, parasite resistance to environmental changes, and membrane recognition and function events. The ΔGDP-MP promastigotes, as a population, display a heterogeneous mixture of long and stumpy forms. Some have normal flagella, while the flagella of some are variably truncated or missing altogether.

The movement of the flagella and that of the promastigotes themselves seems to be more sluggish, in particular those with abnormal flagella (see Supplementary data). An important role for the flagellum as a molecular organiser of cell shape, cytokinesis and cell division in trypanosomes has recently been described (Kohl et al., 2003). The mechanism by which the loss of mannose-containing glycoconjugates affects the flagellar integrity or function is not clear, nor is it clear whether flagellar changes are the cause of the disturbed morphology and cytokinesis of the mutants.

The mutant parasites form large colonies on plastic, but it is not clear whether this is caused by a defect in daughter-cell separation, or increased cell adhesion, or a combination of both. However, the fact that trypsin could disrupt the clumps suggests that cytoadherence caused by protein–protein interactions may be a major factor in the mutant phenotype. Defects in cell division, daughter-cell separation and cytokinesis have also been observed in yeast species in which *GDP-MP* has been down regulated (Warit et al., 2000).

In addition to the altered morphology, the ΔGDP-MP promastigotes exhibit slower growth kinetics compared to wild type parasites in in vitro culture, possibly due to a combination of problems in cell division and nutrient acquisition. The growth arrest at lower density was particularly interesting since cessation of growth is associated with metacyclogenesis and differentiation into the virulent form of the parasites (Sacks and Perkins, 1984). This developmental stage is accompanied by a remodelling of the glycocalyx and extension of the mannose-rich glycoconjugates such as LPG (Sacks, 1989).

The in vitro macrophage infection studies were undertaken in order to shed more light on the parasite defect responsible for the avirulent phenotype. Changes in glycosylation of membrane proteins and glycolipids may be expected to lead to major changes in molecular recognition events and cell–cell interactions. Our studies indicate that despite attaching avidly to macrophages, the ΔGDP-MP parasites were phagocytosed more slowly than the wild type parasites. Video microscopy of the interaction between the parasites and the macrophages showed that wild type promastigotes induced rapid and active movement of the macrophage membrane with ruffling and pseudopod formation. In contrast, the interaction with the mutant parasites led to less ruffling and pseudopod formation. This may be due to their inability to bind to the macrophage CR3, a major host receptor for the wild type *L. mexicana*. Despite the use of a different host receptor for entry into the host macrophage, the ΔGDP-MP parasites localised to the phagolysosome, as did the wild type organisms. However, unlike the wild type parasites, the mutants were killed rapidly within this compartment of the host cell.

The lower efficiency of phagocytosis combined with their hypersensitivity to complement may be significant contributors to the loss of virulence of the mutant parasites in vivo, because promastigotes that are not rapidly phagocytosed are killed in the extracellular environment (Handman, 1999). Hypersensitivity to complement has also been described for mutants lacking some of the surface glycoconjugates such as LPG. The LPG present on the wild type promastigotes, but absent in the mutants, may block access to complement, but additional mechanisms yet to be elucidated may also contribute to the susceptibility of these mutants (Späth et al., 2000, 2003).

There is a large body of evidence that glycosylation increases the stability of some proteins and protects against denaturation (Dwek, 2001). Our observations that the mutant parasites are more sensitive to complement lysis and to alterations in temperature and pH may reflect altered stability of certain proteins that are normally glycosylated. In addition, oligosaccharides may play important roles in targeting of particular proteins in the endoplasmic reticulum and the Golgi apparatus.

Recent studies by Ralton et al. (2003) have shown that the ΔGDP-MP parasites lack a normally abundant intracellular β1-2 mannan which may protect wild type promastigotes from stress, and may account for the susceptibility of the mutants to heat and low pH. In a variety of organisms, including yeast, fungi, insects and plants, other simple sugars such as sucrose and trehalose have been shown to protect proteins from denaturation and to serve as signalling molecules and as sources of energy (Elbein et al., 2003).

The death of the mutant parasites was preceded by the emergence of large vacuoles throughout the cytoplasm and the formation of extremely dense parasite aggregates. The nature of these vacuoles is not yet known, but they are distinct from the very small vesicles detected in sphingolipid-null mutants, which are probably derived from the multi vesicular tubules (Zhang et al., 2003).

The kinetics of death of the ΔGDP-MP parasites were significantly faster in vivo than in vitro, suggesting that the avirulent phenotype may be caused by a combination of the parasites' hypersensitivity to temperature and an increased sensitivity to microbicidal effector molecules such as complement present in the host extra and intra-cellular environment.

The ΔGDP-MP parasites are, to our knowledge, the first lethal temperature sensitive *Leishmania* mutant. Since they were taken up by macrophages and targeted to a cellular compartment compatible with antigen presentation, we reasoned that they should be a good candidate for a live attenuated vaccine (Titus et al., 1995; Veras et al., 1999). Indeed, in two independent experiments, vaccination of BALB/c mice with live ΔGDP-MP promastigotes conferred significant protection from disease. In one experiment, no lesions were observed in the vaccinated mice and in a second experiment the size of the lesions was significantly smaller than controls. In addition, the vaccinated mice harboured a significantly lower parasite burden compared with controls. This effect was only observed when the mice were immunised i.p., suggesting that the route of immunisation may be important. In an early experiment,

s.c. vaccination with live ΔGDP-MP parasites into the footpads of BALB/c mice also led to significantly smaller lesions (a mean of 6 mm) compared with controls (130 mm) 20 weeks after challenge infection with virulent *L. mexicana* (Garami and Ilg, data not shown). Although described before, the mechanism responsible for the effect of the route of immunisation has not been determined (Mitchell et al., 1984, 1985). In contrast to live organisms, killed parasites conferred no protection irrespective of the route of immunisation. Similar results have been described in the *Leishmania major* system (Mitchell et al., 1985). Interestingly, while vaccination with live parasites induced Th1-type immune responses as determined by the ratio of IFN-γ:IL-10, injection of the killed parasites had no such effect (data not shown). Unlike the situation here, in the *L. major* mouse model, s.c. vaccination with an attenuated gene deletion mutant lacking LPG showed excellent protection (Späth et al., 2003). However, it is likely that protection was due to the continuous cryptic persistence of the LPG null parasites (Späth et al., 2003). This is not the case with the totally avirulent ΔGDP-MP which are killed rapidly. Studies are in progress to optimise the ΔGDP-MP as an attenuated live vaccine in a model of infection resembling more closely the natural infection in humans, using a low-dose challenge infection with metacyclic promastigotes in the ear dermis. In addition, we will also examine newly derived *L. major* rather than *L. mexicana* ΔGDP-MP parasites in the *L. major* model. This should provide more information on their protective capabilities and the immune responses that they elicit.

5. Supplementary video

Video microscopy was used to examine the motility of the ΔGDP-MP in comparison to the wild type parasites and in order to analyse the interaction of the ΔGDP-MP parasites with host macrophages.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2005.03.008

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