# *Leishmania mexicana amazonensis*: Plasma membrane adenine nucleotide translocator and chemotaxis

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## Abstract

*Leishmania* cannot synthesize purines de novo and rely on their host to furnish these compounds. To accomplish this, they possess multiple purine nucleoside and nucleobase transporters. Subcellular fractionation, immunohistochemical localization with anti-adenine nucleotide translocator (ANT) antibodies and surface biotinylation show that the mitochondrial ANT is also present in the plasma membrane of both promastigotes and amastigotes. *Leishmania*, however, do not appear to rely on this transporter to supplement their purine or energy requirements via preformed ATP from its host. Rather, *Leishmania* appear to use the plasma membrane ANT as part of a chemotaxis response. ATP is a chemorepellent for *Leishmania* and cells treated with atractyloside, an inhibitor of ANT, no longer exhibit negative chemotaxis for this compound.
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## 1. Introduction

*Leishmania* are flagellated protozoa belonging to the family Trypanosomatidae. They possess a single mitochondrion which undertakes many of the functions found in the mitochondria of higher eukaryotes. Among these functions is the oxidative phosphorylation of ADP by ATP synthase. Because the inner mitochondrial membrane, unlike the outer membrane, is impermeable to ADP and ATP, the mitochondria also possess adenine nucleotide translocator (ANT), an anti-port transporter whose function is to exchange matrix ATP for cytoplasmic ADP (Klingenberg, 1981; Klingenberg and Aquila, 1982). ANT is one of the most abundant integral inner membrane proteins of mito-

chondria comprising about 10–15% of the inner mitochondrial membrane proteins in mitochondria from tissues with high energy requirements, such as heart and muscle (Klingenberg, 1980, 1985; Riccio et al., 1975).

In addition to transporting ADP and ATP across the inner mitochondrial membrane, ANT is also a core component of the mitochondrial permeability transition pore complex (MPTP) which plays a major role in apoptotic cell death (Brustovetsky and Klingenberg, 1996). This protein complex is found in the contact sites between the outer and inner mitochondrial membranes and acts as a nonspecific channel, allowing the free passage of molecules under 1500 Da through the inner mitochondrial membrane (Zamora et al., 2004). In mammalian cells, the major components of the MPTP are ANT in the inner membrane of mitochondria, cyclophilin-D in the matrix and the voltage-dependent anion channel (VDAC) in the outer membrane. The MPTP in *Leishmania* is uncharacterized but this organism also appears to undergo apoptosis or pro-

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grammed cell death (Das et al., 2001; Ouaissi, 2003; Paris et al., 2004; Raina and Kaur, 2006; Shaha, 2006).

*Leishmania* may have a third important role for ANT. *Leishmania* cannot synthesize purines de novo and must rely on their host to satisfy this need (Marr and Bray, 1985). We will show in this communication that in addition to the ANT in the mitochondrion, *Leishmania* also possess ANT in the plasma membrane. However, in contrast to other intracellular parasites which have ANT or ANT like transporters in their plasma membrane, *Leishmania* do not appear to use the plasma membrane ANT to supplement their purine requirements to a significant extent. Instead, the data suggest that the plasma membrane ANT is a component of a negative chemotactic process to delay phagocytosis by neutrophils.

## 2. Methods

### 2.1. Cell culture

*Leishmania mexicana amazonensis* (LV78) promastigotes were grown in M199 medium supplemented with 25 mM HEPES (pH 7.4) and 5% (v/v) heat inactivated fetal calf serum. Amastigotes were grown at 35 °C in J774G8 cells in M199 medium supplemented with 10% (v/v) heat inactivated fetal calf serum and 25 mM HEPES (pH 7.4) and purified on discontinuous Percoll gradients (Chang, 1980). *Leishmania* were transformed by electroporation as described previously (Detke, 1997; Kerby and Detke, 1993).

### 2.2. Gene cloning

Unless otherwise noted, DNA was amplified with Pfu (Stratagene, La Jolla, CA) or Phusion™ (MJ Research, Watertown, MA) and purified by size fractionation in low melting agarose gels. The region of the gel containing the amplified DNA was excised and the DNA therein phosphorylated with the Blunt End cloning kit (Novagen, Madison, WI) and cloned into the dephosphorylated Eco V site of pBC (Stratagene, LaJolla, CA). For some constructs, restriction sites in the multi cloning sequence of pBC were used for subsequent movement of the cloned PCR product into the final destination plasmid.

To construct ANT tagged at the N terminus with yellow fluorescent protein (YFP), the open reading frame of ANT was first amplified with primers 1 and 2 (Table 1), cloned into pBC as described above and then moved into the BamHI/XbaI sites of pALT-NEO (Laban et al., 1990). The open reading frame of YFP without a stop codon was amplified with primers 4 and 5, cloned into pBC and then moved into the BamHI site immediately upstream of ANT in pALT-NEO using BamHI and BglII.

To construct ANT tagged with YFP at the C terminus, the open reading frame of ANT without a stop signal was amplified with Pfu and primers 1 and 3, cloned into pBC and then moved into the BamHI/XbaI sites of pALT-

Table 1
Primers for DNA amplification and cloning[a]

| ANT[b] | | |
|---|---|---|
| 1. | Forward | ggatccATGTCTGCCAACAATAACTCT |
| 2. | Reverse | gtcgactctagaCTAATTCTTCGCCGCGCGAGCACT |
| 3. | Reverse | tctagaagatctATTCTTCGCCGCGCGAGCACT |
| YFP[c] | | |
| 4. | Forward | agatctATGGTGAGCAAGGGCGAGGAG |
| 5. | Reverse | ggatccCTTGTACAGCTCGTCCATGCC |
| 6. | Reverse | tctagaTTACTTGTACAGCTCGTCCAT |

[a] Uppercase nucleotides are for targeting the desired region of the gene. The lowercase nucleotides are for cloning purposes.
[b] Targeting sequence is based on the *L. mexicana* ANT sequence AY187877.
[c] Targeting sequence is based on the GFP in pEGFP-C1 (Clontech, Palo Alto, CA).

NEO. The open reading frame of YFP was then amplified with primers 4 and 6 and cloned into pBC. The YFP open reading frame was released from pBC with BamHI and XbaI and cloned into the BglII and XbaI sites of pALT-NEO/ANT (minus stop signal).

### 2.3. Antibody production

Rabbit anti-ANT antibodies were elicited against the peptide RTRLANDTKSAKKGGERQYS by ProSci, Inc, (Poway, CA).

### 2.4. Subcellular fractionation

Plasma membrane vesicles were prepared by freezing/thawing coupled with discontinuous sucrose gradient centrifugation (Zilberstein and Dwyer, 1988). The purified vesicles were resuspended in 10 mM Tris (pH 6.5), 3 mM MgCl$_2$ and 10 μg/ml leupeptin and stored at −70 °C for immunoprecipitation and immunohistochemical localization experiments. Vesicles used for Western blot analysis were boiled in 2% (w/v) SDS for 2 min and stored at −20 °C. Protein levels were determined with BCA Protein Assay reagent (Pierce Chemical Co., Rockford, IL).

Mitochondrial vesicles were isolated by hypotonic lyses and Percoll gradient centrifugation (Harris et al., 1990). Mitochondrial vesicles used for Western blotting were boiled in 2% (w/v) SDS for 2 min and stored at −20 °C. For immunoprecipitation and immunohistochemistry, the vesicles were suspended in storage buffer (50% (v/v) glycerol, 0.25 M sucrose, 10 mM Tris–HCI (pH 7.5), 1 mM EDTA and 0.1 mM PMSF) and stored at −70 °C.

To isolate the cytoplasmic fraction, *Leishmania* were suspended in 10 mM Tris (pH 8), 2 mM EDTA and 10 μg/ml leupeptin and subjected to 2–3 rounds of freezing/thawing in an −80 °C ethanol bath as well as homogenization with a Dounce homogenizer until the bulk of the cells was disrupted. Unbroken cells, nuclei, cell debris and mitochondria were removed by centrifugation for 15 min at 16,000g in a Sorvall SS-34 rotor. Microsomes were removed by centrifu-

gation at 100,000g for 1 h in the Beckman L8–80 with a 70 Ti rotor. The supernatant from this last centrifugation represented the soluble cytoplasmic fraction.

## 2.5. Surface protein biotinylation

Log phase *Leishmania* were harvested at 2000g and washed three times with Dulbecco's phosphate buffered saline without magnesium or calcium (DPBS). The cells were incubated at $1 \times 10^8$/ml for 1 h at 24 °C with 1 mg/ml EZ-Link™ sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) in DPBS. After the reaction was stopped by the addition of 1/10 volume of 1 M Tris (pH 7.4) for 15 min, the cells were collected at 2000g, washed once with DPBS and extracted at 4 °C twice for 15 min each with 500 µl 100 mM NaCl, 10 mM Tris (pH 7.4), 1% (v/v) Triton X-100, 1× Complete™ protease inhibitors (Roche, Indianapolis, IN). The insoluble material was removed by centrifugation at 16,000g for 15 min at 4 °C. The supernatants were combined and incubated with rotation with immobilized avidin overnight. The beads were collected by centrifugation at 5000g for 10 s and washed three times with extraction buffer. The bound protein was eluted with 1% (w/v) SDS at 70 °C for 10 min.

## 2.6. Western blots

To prepare whole cell lysates, log phase *Leishmania* were harvested at 2000g, washed once with 150 mM NaCl, resuspended in a small volume of 2% (w/v) SDS and boiled for 2 min to inactivate proteases. After protein levels were determined with BCA Protein Assay reagent (Pierce Chemical Co., Rockford, IL) the samples were lyophilized and dissolved at 4 µg/ml in sample buffer (62 mM Tris (pH 6.8), 2% (w/v) SDS, 5% (v/v) 2–mercaptoethanol, 10% (v/v) glycerol). The proteins were separated on a 4–20% PAGEr® polyacrylamide gel (Cambrex BioScience Inc., Walkersville, MD) under denaturing conditions and electroblotted onto PVDF membrane (Harlow and Lane, 1988; Laemmli, 1970). The membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in TBS (20 mM Tris (pH 7.5), 500 mM NaCl) and then incubated for 1 h with the primary antibody and for 1 h with the secondary goat anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase in 1% (w/v) nonfat dry milk, TBS, 0.2% (v/v) Tween 20. The blots were washed three times with TBS, 0.2% (v/v) Tween 20 after each antibody. Horseradish peroxidase activity was detected with the chemiluminescence substrate, SupraSignal West Dura (Pierce Chemical Co., Rockford, IL) and the emitted light captured with a cooled CCD camera (LumiImager, Boehringer Mannheim, Indianapolis, IN).

## 2.7. Mitochondria labeling with MitoTracker® Red 580

Approximately $10^7$ *Leishmania* were harvested at 2000g, washed twice with Dulbecco's phosphate buffered saline without magnesium and calcium (DPBS), and incubated at $10^7$ cells/ml with 250 nM MitoTracker® Red 580 (Molecular Probes, Eugene, OR) in DPBS for 1 h at 24 °C. The cells were washed three times with DPBS and observed within 30 min immediately thereafter by confocal microscopy.

## 2.8. Immunohistochemistry

*Leishmania* were washed three times with Dulbecco's phosphate buffered saline without magnesium or calcium (DPBS), allowed to adhere for 30 min to coverslips which had been pretreated with 0.125 mg/ml poly-L-lysine for 30 min and fixed with freshly prepared 0.5% (w/v) gluteraldehyde in DPBS at 4 °C for 10 min or with 4% paraformaldehyde in DPBS at room temperature for 10 min. To remove excess gluteraldehyde and paraformaldehyde after fixation, the cells were washed three times with DPBS for 5 min per wash. The coverslips were blocked with 1% (w/v) BSA in DPBS for 1 h at room temperature, incubated for 1 h with primary antibody and then for 1 h with secondary donkey anti-rabbit conjugated to FITC or donkey anti-mouse conjugated to TRITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). All antibodies were diluted 1:300 in DPBS, 1% (w/v) BSA. The fixed cells were washed three times with DPBS for 5 min per wash after each antibody.

## 2.9. Fluorescence microscopy

Live cells were photographed under epifluorescence microscopy (Nikon TE300 attached to a Hamamatsu digital camera) through an enhanced GFP filter set. Images were acquired with MetaMorph (Universal Imaging Corp., Downingtown, PA).

Confocal microscopy was conducted with the Olympus IX70 Fluoview 300 laser scanning biological microscope using a 100× oil immersion objective. Green and yellow fluorescent fluorochromes were excited with an argon laser at 488 nm with a detection range of 510–530 nm. Red fluorochromes were excited with a HeNe laser at 543 nm with a detection range of 543 nm and above. Images were captured with the Fluoview software packaged with the Fluoview 300. Figures were composed with Adobe PhotoShop 7.0.

## 2.10. ATP transport

Transport was assessed by the oil stop method (Aronow et al., 1987). $(\gamma^{32}P)$ATP (3000 Ci/mmole) was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). In some experiments the HEPES–RPMI was replaced with 150 mM NaCl, 10 mM HEPES, 10 mM glucose (pH 7.5).

## 2.11. Chemotaxis

Chemotaxis was assessed by the glass capillary method with the following modifications (Leslie et al., 2002;

Oliveira et al., 2000). The 1 mm internal diameter capillary tubes were replaced with the larger diameter Wiretrol II capillary tubes (100–200 μl size, Drummond Scientific Co., Broomall, IL) and the attractant solute was dissolved in 0.4% (w/v) agarose in which the WIS buffer was replaced with cytomix electroporation buffer (van den Hoff et al., 1992). Silicone rubber was used to seal the end of the capillary tube so that the agarose would not slip out of the tube and to prevent dehydration. For the chemotaxis experiments, early stationary phase cells were washed three times at 2000g for 10 min with cytomix electroporation buffer and resuspended at the original cell density.

To determined cell motility, *Leishmania* were washed as described above and placed in a haemocytometer with the attractant solute. Bovine serum albumin was added to 0.4% (w/v) to minimize adherence to the glass. After 10 min at room temperature, the cells were examined under phase contrast microscopy. Cells were considered to be motile if either the flagellum or cell body visible moved over an approximately 15 s observation period.

## 3. Results

### 3.1. Localization of YFP tagged adenine nucleotide translocator

The adenine nucleotide translocator (ANT) is a transporter located in the inner mitochondrial membrane of eukaryotes. To determine if ANT resided in a similar location in *Leishmania*, ANT was tagged with yellow fluorescent protein (YFP) at the N or C terminus and its location ascertained by confocal microscopy (Fig. 1). Panels B and E show a similar internal distribution for ANT whether the YFP resided at the N or C terminus suggesting that the fluorescent protein tag did not alter the targeting of ANT. To verify that this internal location was mitochondria, the ANT–YFP transformed *Leishmania* were incubated with MitoTracker® Red 580, a fluorescent compound which is preferentially sequestered in mitochondria. The merged images of ANT–YFP and MitoTracker® Red showed that the two fluorescent signals overlapped in almost all regions (Panel J). Panels M and O show that there was no ''bleed through'' from these two fluorescent channels.

### 3.2. Co-localization of adenine nucleotide translocator with mitochondrial marker proteins in purified mitochondria and the detection of adenine nucleotide translocator in plasma membrane

To verify the mitochondrial location for ANT, Western blots of purified mitochondrial vesicles were probed with anti-ANT and antibodies against other mitochondrial marker proteins (Fig. 2). Antibodies for the mitochondrial proteins adenylate kinase II (AKII), an intermembrane space protein, and p18, the b subunit of the F0 portion of ATP synthase located in the inner mitochondrial membrane, showed a very strong signal in the mitochondrial fraction
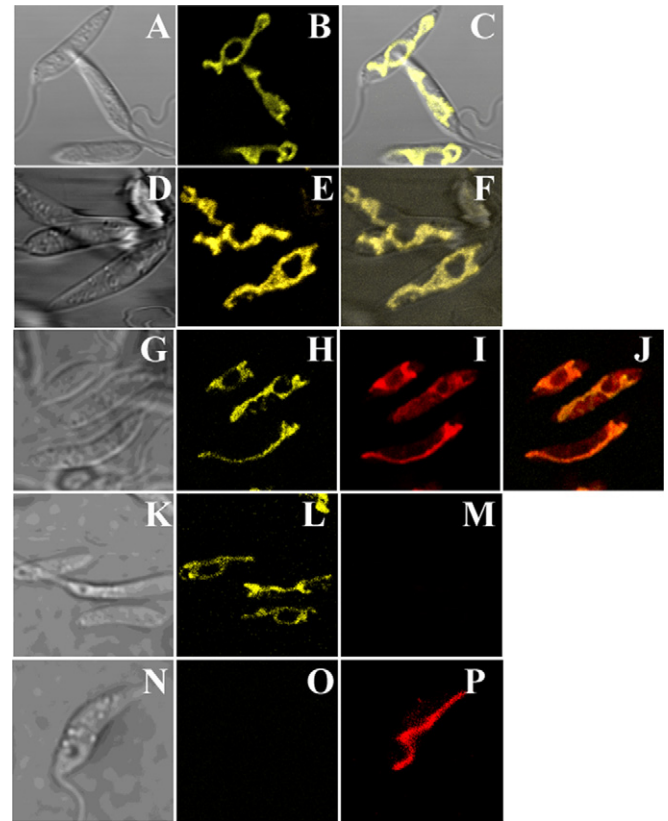


Fig. 1. Localization of YFP tagged adenine nucleotide translocator in *Leishmania*. *Leishmania* transformed with YFP–ANT or ANT–YFP were counterstained with MitoTracker® Red 580 and observed by confocal microscopy. Panels A–C and G–M, *Leishmania* transformed with the ANT–YFP gene; panels D–F, *Leishmania* transformed with the YFP–ANT gene; panels N–P, wild-type *Leishmania*. Panels A, D, G, K and N, differential interference contrast (DIC) microscopy; panels B, E, H, L and O, yellow fluorescence channel; panels I, M and P, red fluorescence channel; panels C and F, merged image of yellow and DIC images; panel J, merged image of yellow and red channels. The cells in panels G–J and N–P were counterstained with MitoTracker® Red 580.

and a much weaker signal in the plasma membrane and cytoplasmic fractions presumably due to a slight mitochondria contamination in these two fractions. Anti-ANT antibodies showed that ANT was also present in the mitochondrial fraction.

Antibodies against gp63, a metalloprotease found in the plasma membrane, yield a weak signal in the mitochondrial fraction indicating that there was a slight contamination with plasma membrane. Purified plasma membrane, on the other hand, showed a very strong signal for gp63 as expected. In contrast to the weak signal for AKII and p18 in the plasma membrane fraction as compared to their more intense signal in the mitochondrial fraction, the signal for ANT in the plasma membrane fraction was much stronger, too strong to merely be due to the trace levels of mitochondrial contamination.

A comparison of the yield of ANT, AKII and p18 indicated that ANT was present in the plasma membrane fraction at a much greater level than were the other two mitochondrial proteins (Table 2). If the amount of p18,
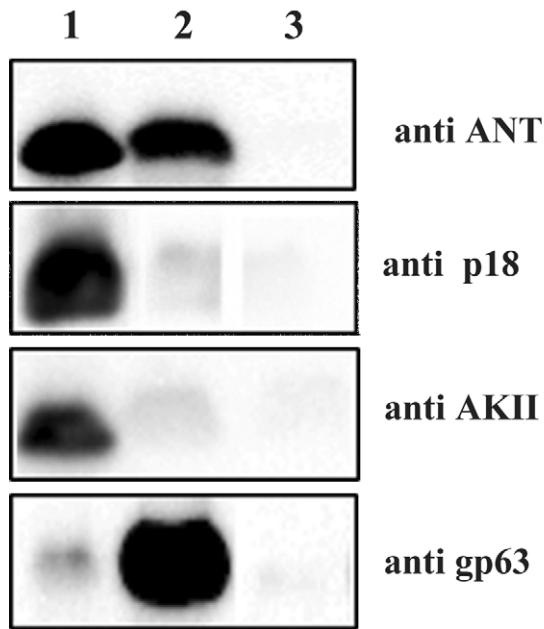
Fig. 2. Subcellular localization of adenine nucleotide translocator in *Leishmania* promastigotes determined by subcellular fractionation. Western blots of mitochondria, plasma membrane and cytoplasmic fractions were probed with the antibodies indicated adjacent to each panel. Equal amounts of protein (5 μg/lane) from the three fractions were resolved by denaturing poly acrylamide gel electrophoresis and blotted onto PVDF membrane. The results are representative of at least six separate experiments. Lane 1, mitochondria; lane 2, plasma membrane, lane 3, cytoplasm.

the b subunit of the F0 portion of ATP synthase which is present in the inner mitochondrial membrane with ANT, is used to estimate the level of mitochondrial contamination, then the yield of ANT in the plasma membrane fraction is about 0.39% of the total ANT (i.e., $0.55 - 0.16\% = 0.39\%$). Using the 2.35% yield for gp63 to estimate the loss of plasma membrane during purification, we estimate that approximately 17% of *Leishmania* ANT would be in the plasma membrane.

The level of adenylate kinase II may not be a reliable indicator of the amount of mitochondrial contamination. AKII is located in the mitochondrial intermembrane space (Nobumoto et al., 1998). Because AKII is not an integral membrane protein and is easily lost with damage to the outer mitochondrial membrane, it may be lost at a greater rate than proteins integrated in the membranes as the mitochondria are fragmented during the purification procedure. The yield of AKII is also much less than the membrane bound mitochondrial markers in purified mitochondria which is consistent with an enhanced loss of this protein during mitochondrial fragmentation (personal observation).

### 3.3. Immunohistochemical verification of adenine nucleotide translocator in the plasma membrane

To verify that ANT was in the plasma membrane, we examined the location of ANT immunohistochemically

Table 2
Purification table showing the levels of target proteins in purified plasma membrane fractions[a,b]

| Whole cell lysate | 1000 mg | |
| --- | --- | --- |
| Plasma membrane | 1.30 ± 0.88 mg | |
| Target protein | Yield (%) | Purification (fold) |
| ANT | 0.55 ± 0.21[c] | 2.52 ± 0.42 |
| P18 | 0.16 ± 0.05 | 0.87 ± 0.42 |
| AKII | 0.07 ± 0.06 | 0.42 ± 0.2 |
| Gp63 | 2.29 ± 0.97 | 14.0 ± 6.63 |

[a] Western blots were carried out as described in Section 2 with equal amount of protein loaded per lane. The light emitted from the chemiluminescence substrate, SupraSignal West Dura, was captured and quantified with a cooled CCD camera (Lumi-Imager). The yield and purification of these four proteins were determined from the signal intensities from the Western blots and the amount of protein in each fraction.
[b] Data are the means ± standard deviation of at least four experiments each performed in triplicate.
[c] Differences between the yield of ANT and p18 were statistically significant as determined with Student's t-test ($p < 0.01$).

with anti-ANT antibodies without intentional permeabilization (Fig. 3). *Leishmania* fixed with paraformaldehyde and probed with anti-ANT antibodies showed ANT internally in mitochondria. The paraformaldehyde fixation apparently permeabilized the cells since anti-carnitine palmitoyl transferase (CPT) antibodies also labeled the mitochondria in these fixed cells. CPT is a mitochondrial protein present in the outer mitochondrial membrane (Bergstrom and Reitz, 1980). The paraformaldehyde did not cause a change in the distribution of plasma membrane epitopes as gp63 and lipophosphoglycans (LPG) were still detected on the periphery of the cells as expected for plasma membrane markers (King et al., 1987; Russell and Wilhelm, 1986). Glutaraldehyde fixation at low temperature, on the other hand, did not yield any signal with the anti-CPT antibodies indicating that this fixation protocol did not permeabilize the cell. With cells fixed with glutaraldehyde, the anti-ANT antibodies yield a punctate fluorescence distributed over the periphery of the cell body and flagellum.

### 3.4. Surface biotinylation of adenine nucleotide translocator

The preceding immunohistochemical localization experiment indicated that ANT was also located on the plasma membrane. The anti-*Leishmania* ANT antibody used in these experiments was very specific for ANT as Western blots of *Leishmania* whole cell lysates yielded a very strong signal in the position expected for ANT and a few very faint bands for larger proteins that were at the limit of detection (personal observation). To eliminate the possibility that the surface protein recognized by the anti-ANT antibodies was a different protein which merely shared the adenine nucleotide translocator epitope recognized by the anti-ANT antibodies, we also labeled the surface proteins with the membrane impermeable biotinylation
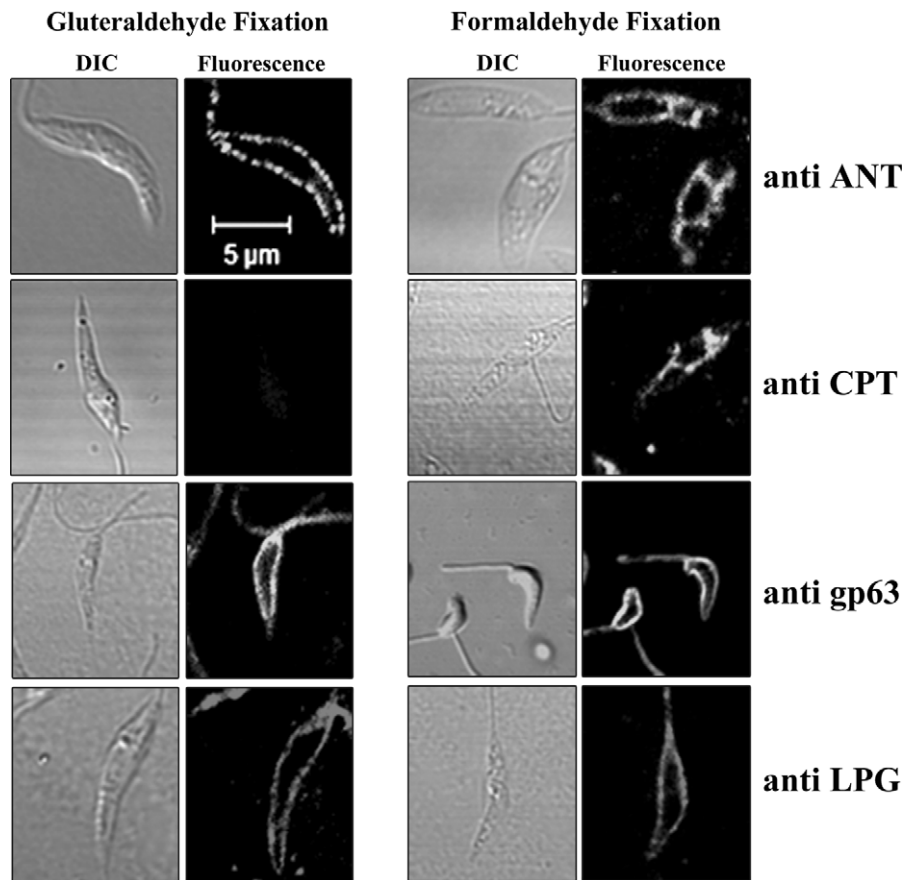
Fig. 3. Localization of the adenine nucleotide translocator in the plasma membrane by indirect immunofluorescence. Promastigotes were fixed with either 0.5% (w/v) gluteraldehyde or 4% (w/v) paraformaldehyde as described in Section 2. The fixed cells were probed with the antibody indicated adjacent to each panel. Polyclonal antibodies used to analyze ANT, CPT and gp63 were visualized with FITC-labeled goat anti-rabbit secondary antibody whereas anti-LPG monoclonal antibody was visualized with rhodamine-labeled goat anti-mouse secondary antibody. DIC, differential interference microscopy.

reagent, EZ-Link™ sulfo-NHS-LC-biotin, and analyzed by Western blots the biotinylated proteins retained on immobilized avidin (Fig. 4). Present in the eluted fraction of cells treated with the biotinylation reagent but absent in the eluted fraction of cells with the mock biotinylation was gp63 as expected because of its plasma membrane location. Also present in the biotinylated material but only present in much lower amount in the mock biotinylated sample was ANT. The presence of ANT in the mock biotinylated sample is probably due to a slight nonspecific absorption to the avidin beads. The biotinylated ANT was not from the mitochondria since AKII, which is found in mitochondria, was not bound by these beads presumably because it was not biotinylated. These biotinylation experiments showed that portions of ANT were exposed to the external environment.

### 3.5. ATP transport by Leishmania

The surface location of ANT suggested that ANT may be used by *Leishmania* as part of a multi-component transport system for the uptake of extra cellular purines from its environment. *Leishmania* cannot synthesize purines de
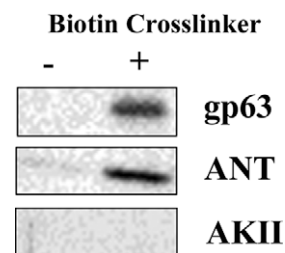


Fig. 4. Biotinylation of plasma membrane adenine nucleotide translocator by a membrane impermeable biotinylation reagent. Late log phase *Leishmania* promastigotes were incubated with the membrane impermeable EZ-Link™ sulfo-NHS-LC-biotin for 1 h and processed as described in Section 2. Biotinylated proteins were isolated by chromatography on immobilized avidin columns and analyzed by Western blotting with the antibodies indicated adjacent to each panel. +, cells with biotin cross linker; −, cells without biotin cross linker.

novo and must rely on its host to satisfy their purine needs. To test this hypothesis, the accumulation of radiolabeled ATP by *Leishmania* was examined. We were unable to unequivocally detect significant accumulation of exogenous ATP. There was a small amount of uptake of radiolabeled ATP over the first 20–30 s that was barely detected above background. The accumulation was temperature sensitive

but was not inhibited by sodium atractyloside, an inhibitor of adenine nucleotide translocator (data not shown). Omitting magnesium in the transport medium to reduce ecto-ATPase activity by replacing the RPMI buffer with HEPES buffered isotonic saline solution did not yield a noticeable increase in the amount of ATP accumulated (Berredo-Pinho et al., 2001; Meyer-Fernandes et al., 1997).

### 3.6. Adenine nucleotide translocator and chemotaxis

The preceding ATP transport experiments indicated that the plasma membrane ANT was not a significant component of the family of plasma membrane purine transporters used by *Leishmania* to satisfy their purine requirement. We then examined a potential role for ANT in the phagocytosis of *Leishmania*. The literature showed that some phagocytic cells (i.e., neutrophils) released ATP at the leading edge which reinforced their movement in that direction (Chen et al., 2006). Neutrophils are particularly interesting as they are the first phagocytic cells to infiltrate the site of infection and function as a temporary home for *Leishmania* before ingestion by macrophages in which they grow very well (van Zandbergen et al., 2004). To ascertain if ATP is a chemoattractant, we used the capillary method instead of the "mean time of straight line movement" described by Barros et al. (2006). We were unable to use the "mean time of straight line movement" method to examine chemotaxis because there was too great of a range of movement among *Leishmania* to identify a representative population and we did not observe the "tumbling" which signaled a change in movement.

*Leishmania* responded to the ATP gradient but instead of migrating toward the source they moved away from the ATP (Fig. 5). The difference in cell density within the capillary tubes with ATP and the control tubes without ATP was statistically significant as determined by Student's *t*-test for ATP concentrations as low as 10 μM. The concentration of ATP diffusing into the reservoir at the head of the capillary tube would have been lower as Oliveira et al. have determined that the concentration of the attractant in the first 0.5 cm of the open extremity of the tube was less than 1/20 of the concentration within the agarose plug (Oliveira et al., 2000). This difference places the effective ATP concentration in the same range as sucrose, lactose, mannitol and glycine albeit as a negative instead of a positive chemotactic compound (Barros et al., 2006). In the experiments shown in Fig. 5, *Leishmania* exhibited positive chemotaxis for glucose as reported in the literature although at this high concentration of glucose they were probably responding more to the osmotic gradient than to the glucose gradient (Leslie et al., 2002; Oliveira et al., 2000).

To verify that *Leishmania* were responding to the ATP gradient, we also examined chemotaxis with ATP added directly to the cells in the lower reservoir in addition to the agarose plug within the capillary tube thereby eliminating the gradient. There was no difference in the cell density
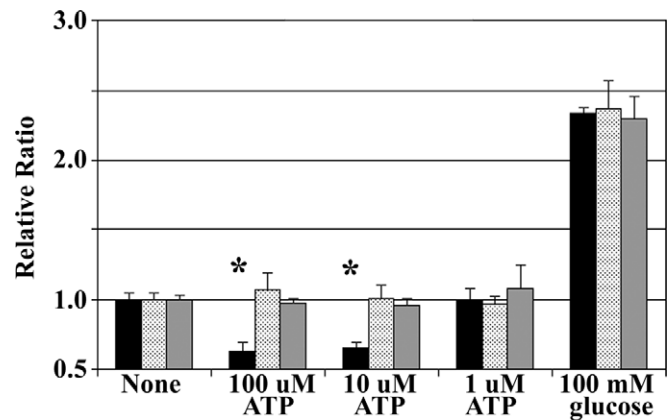


Fig. 5. Chemotactic response of *Leishmania* to ATP. The chemotactic response of *Leishmania* to ATP and glucose was assessed by the capillary method as described in Section 2. The relative ratio is the cell density in the capillary tube with the attractant relative to the density in the capillary tube with no attractant. The data are the means ± standard deviation from two experiments each done in triplicate. The statistical significance was determined by Student's *t*-test: $^*p < 0.01$. Black bar, control cultures; stippled bar, *Leishmania* with 100 μg/ml sodium atractyloside, an inhibitor of ANT; grey bar, *Leishmania* with 10 μM ATP added to the main reservoir of cells.

within the capillary tubes between these cells and the control experiments without any ATP in the lower chamber.

To determine if ANT was involved in this negative chemotaxis, the cells were also exposed during the chemotaxis test period to sodium atractyloside, a competitive inhibitor of ANT (Bruni et al., 1965; Vignais and Vignais, 1964). The sodium atractyloside inhibited the movement away from ATP but not from glucose indicating that different sensors were involved and that ANT played an active role in this negative ATP chemotaxis.

It is possible that *Leishmania* did not migrate away from the ATP but merely stopped moving in the presence of ATP. In vivo, this would result in *Leishmania* being more readily phagocytized by neutrophils, their first hosts in mammals, if they did not swim in all directions. To test this possibility, we incubated *Leishmania* with different concentrations of ATP and counted the number of cells which were motile after a 10 min incubation period. There was, however, no obvious difference in motility of *Leishmania* incubated in the presence or absence of 100 μM or 1 mM ATP (data not shown). There was also no difference in motility for cells incubated in the presence of 100 μg/ml sodium atractyloside with and without 100 μM ATP. These data indicate that sodium atractyloside did not impair motility per se and that ATP is a negative chemotactic compound which does not simply cause the cells to stop moving into the capillary by impairing their motility.

### 3.7. Surface adenine nucleotide translocator in amastigotes

To determine if the adenine nucleotide translocator was also present in the plasma membrane of amastigotes, amastigotes were isolated from the murine macrophage

line, J774G8, fixed with glutaraldehyde in order to maintain the integrity of the plasma membrane to antibodies and probed with anti-ANT antibody (Fig. 6). Anti-ANT antibodies also yield a distinct peripheral signal in this stage. In contrast to relative sparse punctate pattern observed for promastigotes, the fluorescence on amastigotes appeared to cover a larger fraction of the surface.

To verify that the surface protein recognized by the anti-ANT antibodies was adenine nucleotide translocator, we also labeled the surface proteins of amastigotes with the membrane impermeable biotinylation reagent, EZ-Link™ sulfo-NHS-LC-biotin, and analyzed by Western blots the biotinylated proteins retained on immobilized avidin (Fig. 7). ANT was present in the biotinylated material in much higher levels as compared to the mock biotinylated

sample. We suspect that the lower amount of ANT in the mock biotinylated experiments reflects the nonspecific absorption of ANT onto these beads. These biotinylation experiments further demonstrate that portions of ANT in amastigotes were exposed to the external environment.

### 3.8. Comparison of ANT levels in promastigotes and amastigotes

Although amastigotes are smaller than promastigotes, the overall protein concentration is about the same. Promastigotes have approximately five times the volume of amastigotes and five times as much protein per cell (Coombs et al., 1986; Hart et al., 1981). To determine if there were differences between these two stages in the amount
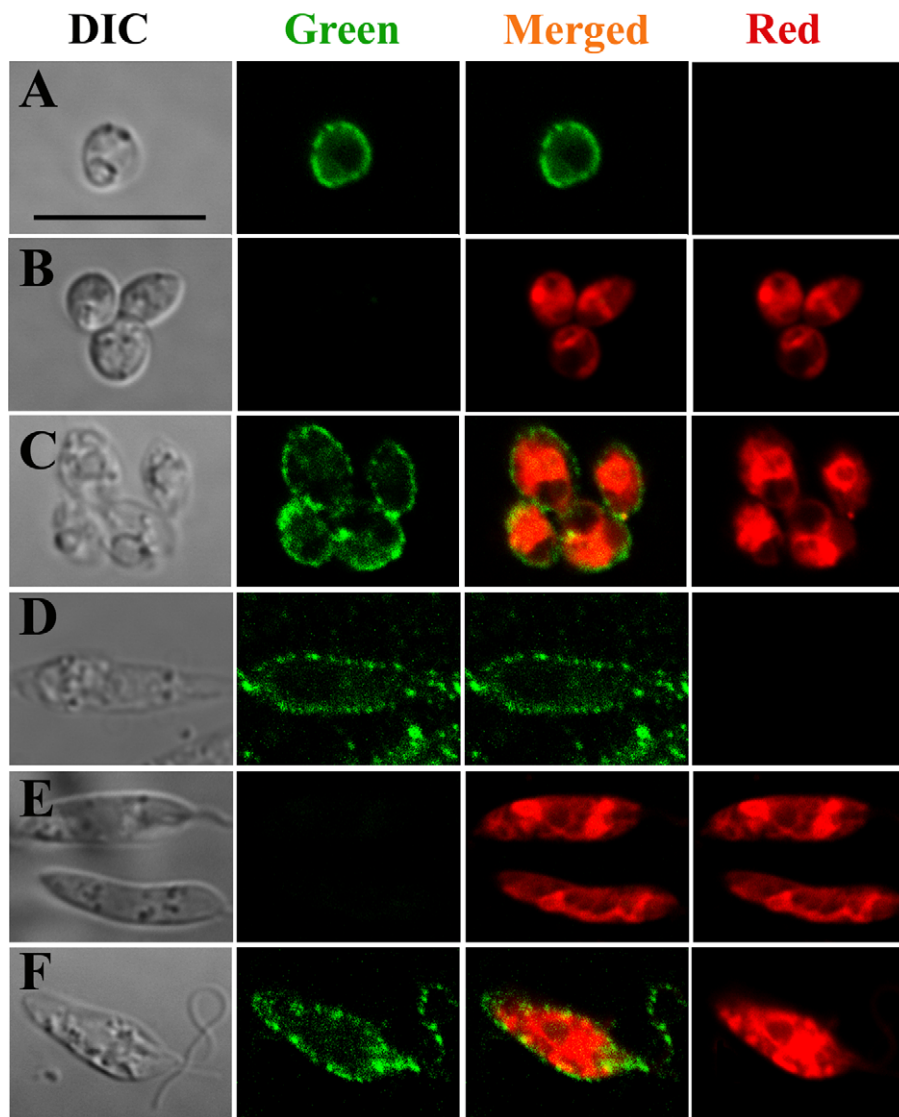


Fig. 6. Localization of the adenine nucleotide translocator on the plasma membrane of amastigotes by indirect immunofluorescence. *Leishmania* were fixed with 0.5% (w/v) gluteraldehyde as described in Section 2 and probed with preimmune or anti-ANT antibody. The distribution of the primary antibodies was visualized with FITC-labeled goat anti-rabbit secondary antibody. Top three panels, amastigotes; bottom three panels, promastigotes. Panels B and E, preimmune serum; panels A, C, D and F, anti-ANT antibody. Panels B, C, E and F, *Leishmania* co-stained with MitoTracker® Red 580. DIC, differential interference microscopy; Green, green fluorescence channel; Red, red fluorescence channel. The bar in panel A is 10 μm.
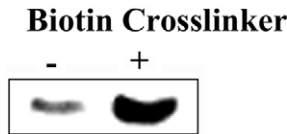
**Biotin Crosslinker**



Fig. 7. Biotinylation of plasma membrane adenine nucleotide translocator in amastigotes by a membrane impermeable biotinylation reagent. *Leishmania* amastigotes were purified from J774G8 cells, incubated with the membrane impermeable EZ-Link™ sulfo-NHS-LC-biotin for 1 h and processed as described in Section 2. Biotinylated proteins were isolated by chromatography on immobilized avidin columns and analyzed by Western blotting with anti-ANT antibodies. +, cells with biotin cross linker; −, cells without biotin cross linker.
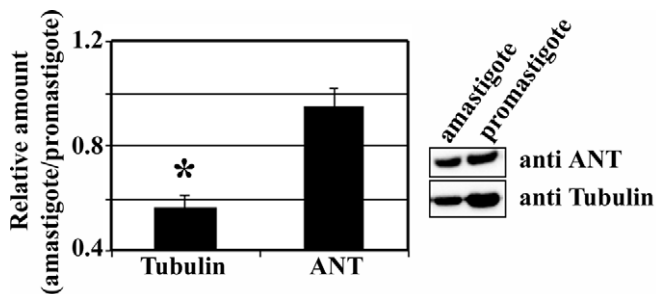


Fig. 8. Comparison of the relative abundance of adenine nucleotide translocator in amastigotes and promastigotes. Western blots of whole cell lysates (i.e., 20 μg total protein per lane) from amastigotes and promastigotes were probed with anti-ANT and anti-tubulin antibodies. The data are the means ± standard deviation ($N = 3$). The difference in ANT levels between amastigotes and promastigotes was not statistically significant as determined by Student's $t$-test ($p > 0.05$) but the difference for tubulin between these two stages was statistically significant (*$p < 0.01$). The Western on the right shows the relative intensity of the bands from amastigotes and promastigotes from one of the above experiments.

of ANT, we determined ANT levels by Western blots. A comparison of the signal intensity from Western blots of whole cell lysates from promastigotes and amastigotes showed no significant differences in the amount of ANT per μg of protein loaded on the gel (Fig. 8). On a per microgram basis, amastigotes had 95 ± 7% as much ANT as did promastigotes. In contrast, amastigotes had 57 ± 5% as much tubulin as did promastigotes. It has been previously shown by others that amastigotes possess reduced levels of tubulin as a result of differences in post-transcriptional or translational regulation of the tubulin mRNAs as compare to promastigotes (Bhaumik and Adhya, 1993; Fong and Chang, 1981; Fong et al., 1984; Joshi et al., 1995; Landfear and Wirth, 1984; Miller, 1988). These data indicate that ANT comprises the same relative amount of protein in both stages.

## 4. Discussion

In this communication we show that *Leishmania* have adenine nucleotide translocator in the plasma membrane in both promastigote and amastigote stages. Cellular fractionation, surface protein labeling and immunohistochem-

istry supported the presence of ANT in this location in addition to the traditional mitochondrial location.

Immunohistochemistry only revealed surface ANT in fixed cells with antibody impermeable membranes. Surface ANT was not observed in fixed cells with antibody permeable membranes. The absence of visible surface ANT in these cells with antibody permeable membranes as well as in nonfixed cells in which the ANT was tagged with green or yellow fluorescent protein may be a detection/sensitivity problem. Less than 20% of the total ANT is present in the plasma membrane and it is distributed, albeit in a punctate fashion, over the entire surface of the cell body and flagellum. The fluorescence intensity from the surface ANT would therefore be much less than the ANT present in the mitochondria. Long exposures to visualize the putative lower abundance surface ANT simultaneously with the higher abundance mitochondrial ANT only yielded badly overexposed images of poor definition and quality whenever mitochondrial ANT was visible (data not shown).

The punctate surface ANT distribution in amastigotes is also present but not usually as obvious as in promastigotes. We estimate that the promastigote surface area is approximately 4- to 5-fold greater than that of amastigote. Since these two stages have approximately the same amount of ANT per unit amount of total protein, we would also expect that the amastigote plasma membrane ANT should exhibit a similar relatively diffuse punctate pattern. Instead the surface is usually more nearly continuously covered. We hypothesize that the ANT originally present on the flagellar membrane may have relocated to the plasma membrane when the amastigote lost its flagellum or there may have been a shift in the degree of targeting of the ANT between mitochondria and plasma membrane. A BLAST search of the *Leishmania* genome at the GeneDB website (http://www.genedb.org) indicated that *Leishmania* has two copies of the ANT gene (i.e., LmjF19.0200 and LmjF19.0210) and both copies code for identical proteins. The increase in the apparent density of ANT on the amastigote plasma membrane could not have been due to increased expression of a plasma membrane specific ANT variant in this stage.

The detection of ANT in the plasma membrane suggested that *Leishmania* may also possess the capability to transport exogenous ATP to supplement their purine or energy requirements. ANT is an anti-port transporter that transports ATP and ADP. The binding of nucleotides to ANT in the mitochondria, induces conformational changes that trigger the one-to-one exchange of ADP with ATP (Duyckaerts et al., 1980). In mitochondria, ANT transports ADP into the matrix and ATP out into the cytoplasm by way of the intermembrane space and outer membrane pores. To accomplish this, ANT is orientated so that the N and C terminus and the other odd numbered, short extramembrane domains reside on the intermembrane or cytoplasmic face of the mitochondria whereas the even numbered, long extramembrane domains reside on the matrix face. The ability of anti-ANT antibodies to detect

ANT in the plasma membrane of fixed, nonpermeabilized *Leishmania* indicates that ANT in the plasma membrane has a similar orientation, odd numbered, short domains facing the cytoplasm and even numbered, long domains facing away from the cytoplasm (i.e., the extracellular environment). The ANT antibodies were raised against a peptide present in loop IV which is located in the mitochondrial matrix. Without permeabilization of the plasma membrane, these antibodies would only have detected ANT if loop IV extended out into the extracellular environment. This orientation positions ANT so that it has the potential of transporting ATP into *Leishmania*.

Although the plasma membrane ANT is in an orientation that positions it to potentially be capable of transporting ATP into *Leishmania*, we have not been able to unequivocally measure this uptake. It is not known if this surface ANT is functional but its ATP transport activity is too difficult to detect because it is too low relative to ecto degradative enzymes which metabolize ATP. *Leishmania* possess a Mg-dependent ecto-ATPase (Berredo-Pinho et al., 2001; Meyer-Fernandes et al., 1997). This ATPase is sensitive to DIDS (4,4′-diisothiocyanostylbene 2′,2′-disulfonic acid) and suramin but even millimolar concentrations of these compounds only partially inhibit its activity. In contrast to ANT which exhibits maximal transport activity in the absence of magnesium, the *Leishmania* ecto-ATPase, in the absence of magnesium, has only 10% of its maximal activity (Barbour and Chan, 1981; Berredo-Pinho et al., 2001; Klingenberg, 1977). We, therefore, tried to measure ATP uptake in medium lacking magnesium but did not detect any increase relative to medium containing magnesium (data not shown). There may be other yet unidentified ecto enzymes which metabolize ATP.

Alternatively, the *Leishmania* plasma membrane ANT may not be very active in this environment. In mitochondria, ANT binds six molecules of cardiolipin per dimer which can only be removed under very harsh conditions (Beyer and Klingenberg, 1985). It may also bind other phospholipids but with lower affinity. Cardiolipin seems to be an activator of this mitochondrial transporter as ANT mutants which readily loose cardiolipin upon purification show no activity in reconstituted membranes unless cardiolipin is added (Liu et al., 2001). In *Leishmania*, cardiolipin comprises 10.5% of the lipid in mitochondria but only 0.6% of the lipid in plasma membrane (Wassef et al., 1985). The true relative abundance of cardiolipin in the plasma membrane may be even less as part of this 0.6% may originate from trace levels of mitochondrial membrane contaminating the purified plasma membrane fraction. There may simply be insufficient cardiolipin present in the *Leishmania* plasma membrane to fully activate plasma membrane ANT.

*Leishmania* is not the only organism which exhibits an atypical location for ANT as ANT and ANT-like transporters have also been shown to be present in the plasma membrane of Plasmodium and fibroblasts and in the membranes of the obligate intracellular bacteria, *Rickettsia*

*prowazekii* and *Chlamydia trachomatis* (Choi and Mikkelsen, 1990; Hatch et al., 1982; Kanaani and Ginsburg, 1989; Resh and Ling, 1990; Schmitz-Esser et al., 2004; Sigal and Resh, 1993; Winkler, 1976). It is not clear, what the function is for plasma membrane ANT in fibroblasts. For intracellular parasites, on the other hand, the plasma membrane ANT appears to contribute to the "energy parasitism" of these organisms, a reliance on their host not only for nutrients but also for a portion of their energy in the form of ATP. Plasma membrane ANT may function in *Leishmania* in a similar capacity albeit not to a major extent since most of the ANT is located in a functional mitochondria and that which is present in the plasma membrane does not appear to be very active.

A biologically more relevant role for the plasma membrane ANT may be as a component of an extracellular ATP sensor. *Leishmania* exhibit negative chemotaxis for ATP and may recognize extracellular ATP as a signal to move away from damaged/dying cells at the site of the sandfly bite which may be leaking ATP. Alternatively, the negative chemotaxis for this compound may be a defensive mechanism enabling *Leishmania* a longer opportunity to adapt to the hostile environment of the phagosome before being phagocytized. Neutrophils are the first phagocytic cells to infiltrate the site of infection and function as a temporary home for *Leishmania* before ingestion by macrophages (van Zandbergen et al., 2004). *Leishmania* do not multiply in neutrophils but do promote the longevity of these cells by delaying their apoptotic death program by approximately two days (Aga et al., 2002). These infected cells are then phagocytized by macrophages in which the amastigotes take up residence and multiply. Neutrophils release ATP at the leading edge which reinforces their movement in that direction (Chen et al., 2006). By sensing the ATP released by the neutrophil and "fleeing" from this leukocyte, *Leishmania* might have a little bit more time to activate/increase the activity of enzymes enabling them to grow in an environment made toxic by reactive oxygen and nitrogen species. Amastigotes are more resistant to hydrogen peroxide and nitric oxide than are promastigotes and are so at least in part because they possess higher levels than do promastigotes of both FeSOD and the peroxidoxin Pxn1, enzymes which are involved with the detoxification of these compounds (personal observation and references Barr and Gedamu, 2001, 2003; Jirata et al., 2006; Paramchuk et al., 1997; Pearson et al., 1983).

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