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The role of protein phosphorylation in differentiation of *Leishmania* parasites

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Part II - Co - principal investigator

Part I - Principal investigator

Executive summary

Leishmania are obligatory intracellular parasites that cause leishmaniasis in Peru and Israel. These organisms cycle between phagolysosomes of human macrophages (the host) and elementary tracts of female sandflies (the vector). Both acidic pH (< 6.0) and elevated temperatures (~ 37^0C) trigger differentiation of *L. donovani* promastigotes to amastigotes. The aim of this project is to elucidate the role of post translational processes such as protein phosphorylation in leishmanial differentiation. Previously, we reported that exposing *L. donovani* promastigotes to both environmental conditions induce rapid phosphorylation and dephosphorylation of cellular proteins, indicating that post translational processes involved in parasite differentiation. In order to reach our goals we decided that it is important to develop methods for growing amastigotes extracellularly in culture. This was achieved by mimicking the environmental conditions in phagolysosomes. The axenic amastigotes resemble the animal-derived amastigotes; they do not synthesize lypophosphoglycan (whereas promastigotes do) and possess amastigotes-like proline transport activity. In parallel, we used PCR to amplify DNA that code for the highly conserved catalytic site of protein kinases, using *L. donovani* genomic DNA as a template. The experiments yielded four different 206 bp DNA pieces that possessed the most of protein kinase conserved regions. Two of these did not resemble any know protein kinase, whereas the other two obtained high level of homology to active sites of cdc2 gene family. One of the latter, A1, was cloned into pQE, expressed at high levels in *E. coli*. The resultant protein was purified and injected into rabbits in order to raise specific antibodies against it. We plan on using these antibodies to identify the corresponding protein kinase in *L. donovani* (in Israel) and *L. peruviana* (in Peru). In parallel we shall use the PCR products as probes to clone their genes from both genomic and cDNA libraries that we have constructed from promastigotes and amastigotes of *L. donovani*. The amplified DNA as well as the specific antibodies will also be transferred to the CO-PI in order for them to identify corresponding protein kinases.

Collaboration: Axenic cultures of amastigotes have been developed in both Peru and Israel. The amplified DNA will be used by both the PI and CO-PI's groups to clone protein kinase genes. In order to do so, a student from the CO-PI's group will come to the PI's laboratory to carry out these experiments. Antibodies against A1 will be used in both groups to identify developmentally regulated protein kinase. The PI and CO-PI plan on visiting each other during 1997.

Section I

A) Research objectives

Previously we reported that acidic pH and elevated temperatures induce protein phosphorylation and dephosphorylation. These environmental factors trigger *in vitro* differentiation of *L. donovani* promastigotes to amastigotes (10). The results indicated that post translational processes are involved in parasite differentiation.

In order to further investigate leishmanial differentiation, we decided to focus on developing methods for growing *L. donovani* amastigotes in axenic cultures, devoid of their host cells. Therefore, the specific objectives were as follow:

1. To device a methods for growing *L. donovani* amastigotes in culture.
2. To identify protein kinase genes in the genome of *L. donovani*.
3. To exploit the *in vitro* cultures to assess the role of protein kinases in the development of *L. donovani*.

B) Research accomplishments

B.1. Establishment of axenic amastigotes in culture.

Axenic amastigotes: In order to investigate post translational processes that regulate differentiation in *L. donovani* it was important to obtain an *in vitro* system in which amastigotes can grow axenically in culture, devoid of their host macrophage. When we started this project, such culture was not available. Therefore, we decided to devise methods for *in vitro* cultivation of *L. donovani* amastigotes. The idea was to mimic the *in situ* physiological conditions inside phagolysosomes. Promastigotes in culture (medium 199) were first shifted from 26^0C to 37^0C with 5% CO_2. After 24 hours, the pH of the growth medium was changed to 5.5. Under these conditions parasites differentiate to amastigotes within 5 days. Fig. 1 illustrates the shape of the various life stages of the *in vitro L. donovani* cultures. When mid-log phase *L. donovani* promastigotes (fig. 1A) are transferred to 37^0C in the presence of 5% CO_2 (Fig. 1C), they form large aggregates that start to appear 8 hours after the temperature shift. This process can be inhibited by the addition of cycloheximide, a protein synthesis inhibitor (Ashamoa and Zilberstein, unpublished results). Note that complete differentiation of promastigotes to amastigotes succeeded only when parasites were exposed to the combination of acidic pH and elevated temperature (fig. 1D). Promastigotes that have been adapted to grow at pH 5.5 without changing the temperature, although rounded up, maintained the shape of promastigotes (Fig. 1B).

To show that the *in vitro* amastigotes resemble animal-derived amastigotes, we set up two experiments, synthesis of lipophosphoglycan and uptake of proline. Both of these activities are developmentally regulated.

Leishmania promastigotes synthesize lipophosphoglycan (LPG) that forms thick cover on the surface of these parasites. Turco and Sacks (6,7) found that synthesis of LPG is stage specific; promastigotes synthesize it but amastigotes do not. Hence, LPG synthesis activity can be utilized as marker for the various life stages of *L. donovani*. LPG in *L. donovani* cultures was metabolically labeled using ^{3}H-mannose. Subsequently, LPG was purified (7) and the level of ^{3}H-mannose was determined. The results of these experiments are summarized in Table 1. Late log phase axenic *L. donovani* promastigotes synthesize large amounts of LPG as indicated

by the high level of incorporation of the radiolabeled mannose. On the other hand, axenic amastigotes hardly synthesize LPG and the rate of incorporation decrease with increase in age of the culture amastigotes.

Second marker for successful *in vitro* differentiation to amastigotes is proline transport. Previous studies indicated that in *L. donovani* promastigotes proline transport activity is optimal at around pH 7 (9), whereas in amastigotes it is optimal at around pH 5.5 (2). As shown in Fig. 2A, proline uptake in *L. donovani* promastigotes was relatively high, reaching maximal activity at around extracellular pH 7.0. On the other hand, axenic amastigotes obtain proline transport activity at much lower level that resemble animal-derived amastigotes, and it is optimal at around pH 5 (Fig. 2B).

The results in Figs. 1 and 2 indicate that the procedure we have devised for *in vitro* differentiation yields axenic amastigotes that are very close to the real animal-derived amastigotes.

Fig. 3 illustrates the pattern of proteins in the various cultures of *L. donovani*. It is clear from the figure that most of the proteins remain unchanged after differentiation. There are a few proteins that appear and others that disappear during the differentiation process. For example, the level of protein 1 increased following parasites' adaptation to growth at 37^0C and reach its maximal level after differentiation to amastigotes completed. Protein 5 is most likely an amastigotes stage-specific protein. On the other hand, proteins 3, 4 and 6 appeared only in promastigotes. Since the proteins in Fig. 3 were stained with coomaassie blue, they represent parasites' proteins at steady state, i.e. after they have established in appropriate conditions and not the rate of synthesis.

In order to assess the changes in protein synthesis that occur right after the induction of differentiation, we carried out short-term metabolic labeling of parasites using ^{35}S-methionine. In the first set of experiments, mid log phase promastigotes were divided into two; one was shifted to grow at pH 5.5 and the second remained at pH 7.0. Both cultures were incubated at 26^0C for one hour and then ^{35}S-methionine was added and incubation continued for another 2 hours. Subsequently, the labeled proteins were separated on 2 dimensional gel electrophoresis, using the non equilibrium pH gradient electrophoresis electrophoresis methods (4) and subsequently exposed to autoradiography. The results of this experiments are summarized in figs. 4 A and B. The results that most of the labeled proteins remain unchanged after the pH shift and only in a few change in the rate of synthesis was observed. For example, the synthesis of proteins 1, 6 and 7 was more rapid at pH 5 than in promastigotes at pH 7. Protein 3 is synthesized only after shifting the medium pH to 5. Hence, growth medium pH has a role in regulating protein synthesis in *L. donovani*.

Next, we looked at changes in protein synthesis that occur after shifting promastigotes from pH 7 at 37^0C to pH 5.5 at 37^0C (conditions that induce differentiation, figs. 5 A and B). A Few proteins such as those marked 5, 9, 10, 12 obtain lighter bands at the amastigotes conditions. Those marked by the white arrows such as 4, 8 and others are enhanced at pH 5 at 37^0C. We failed to observe protein synthesis that could be interpreted as amastigotes stage-specific labeling by ^{35}S-methionine.

<u>*DNA and protein synthesis by axenic promastigotes*</u>. *L. donovani* are sensitive to changes in extracellular pH. They response to acidification of extracellular pH by changing both gene and phenotypic expression (8-10). For example, shifting *L. major* promastigotes from pH 7 to 4.5 induced the expression of an amastigotes stage-specific surface protein (8), as well as change in proline transport activity (9). Furthermore, Mukkada et al. (5) showed that amastigotes are able to adjust their metabolic activities to the environment of the host. Hence, part of the differentiation process most likely involve adaptation to the acidic

environment within the host lysosome. If this is the case then the *in vitro* amastigotes' metabolic activities should resemble that of animal-derived amastigote, as described by Mukkada et al. (5). The following experiments was set up to look at pH response of various metabolic activities in the *in vitro L. donovani* amastigotes.

Figure. 6 illustrates pH response of thymidine incorporation in *L. donovani* cultures. Promastigotes incorporate thymidine best at around pH 7. The *in vitro* amastigotes incorporate thymidine at much lower rate, which is proportional to the difference in growth rate. However, unlike promastigotes, the *in vitro* amastigotes incorporate thymidine best at pH 5, resembling corresponding activity in animal-derived amastigotes (5). Similar adaptation to extracellular pH was also observed for proline incorporation, Fig. 7. Promastigotes incorporate proline at high rate with an optimum activity at pH 7, whereas the *in vitro* amastigotes incorporate proline best at pH 5, but the incorporation activity was 10-fold lower.

The results in figs. 6 and 7 further indicate that the *in vitro* amastigotes resemble the animal-derived amastigotes. The advantage of the *in vitro* cultures of *L. donovani* amastigotes is that by controlling the environmental conditions we can investigate , at the molecular level, processes that play roles in regulating leishmanial development.

B.2. Identification of protein kinase genes.

Amplification of L. donovani pk genes. The observation that protein phosphorylation occur right after the induction of differentiation (previous report) prompted us to look for protein kinases in these parasites. The catalytic region protein kinases is highly conserved throughout evolution, fig. 8 (3). Degenerate oligonucleotide primers from 2 such domains, VIb (IHRDL) and IX (DVWSF) have been synthesized by General Biotechnology Inc. (Rehovot). The sense primer responding to the amino acid IHRDL: 5'-CGGAT(T/C) CACCG(C/G)GACCT - 3' (PKI) while the antisense primer encoding DVWSF: 5' - GGAATTGAA(G/C)(G/C)(T/A)CCA(G/C)ACGGTC - 3' (PKII). These primers were used in a PCR to amplify the potential protein kinase genes from the genome of *L. donovani*. The PCR product ranged from 150 - 206 bp, fig. 9. DNA fragments of the expected size (206 bp) were recovered and subcloned into pGEM-T plasmid (Promega Inc.). Nucleotide sequence of analysis showed that 5 of the cloned PCR products deduced amino acid sequences contain the sequence that corresponding regions of the catalytic domains of serine/threonine protein kinases, suggesting that these sequences were derived from respective protein kinase genes in *L. donovani* genome. Three of these are identical except for 3 amino acids (A1-A3, fig 10). Southern analysis indicated that A1-A3 form identical pattern of hybridization with *L. donovani* genomic DNA, indicating that they belong to the same gene. The other 3 PCR products hybridize with 3 different genes in *L. donovani*, each gene most likely appears as a single copy genes.

A search of the sequence databases revealed that the peptide A1 is most closely related to the cdc2-related family (fig. 11). The highest identity (57%) was to p58 galactosyltransfferase, a protein involved in regulation of cell cycle (1). The other peptides, A4 and A6 although encode for protein kinases, they are not similar to any of the kinases in the database. They could be leishmanial novel kinases.

Since we are interested in protein kinase that play a role in parasite differentiation, we decided to pursue our work with A1 peptide.

Antibodies against A1 protein recognize p34. A1 was subcloned in frame into pQE transcription-translation plasmid (Promega Inc.). The recombinant protein of 7800 kDa (p8 fig. 12) contained 6 histidines at its N-terminus was purified on a nickel affinity column, injected into rabbits in order to raise specific polyclonal antibodies.

B.3. References

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B.4. Tables and Figures

Table 1: LPG synthesis by *in vitro* promastigotes and amastigotes of *L. donovani*.

Parasites	**Incorporation of ^{3}H-mannose into LPG**
	cpm
Late log promastigotes	73900
1 week old amastigotes	2100
5 weeks old amastigotes	800
Log phase of recycled promastigotes	129650

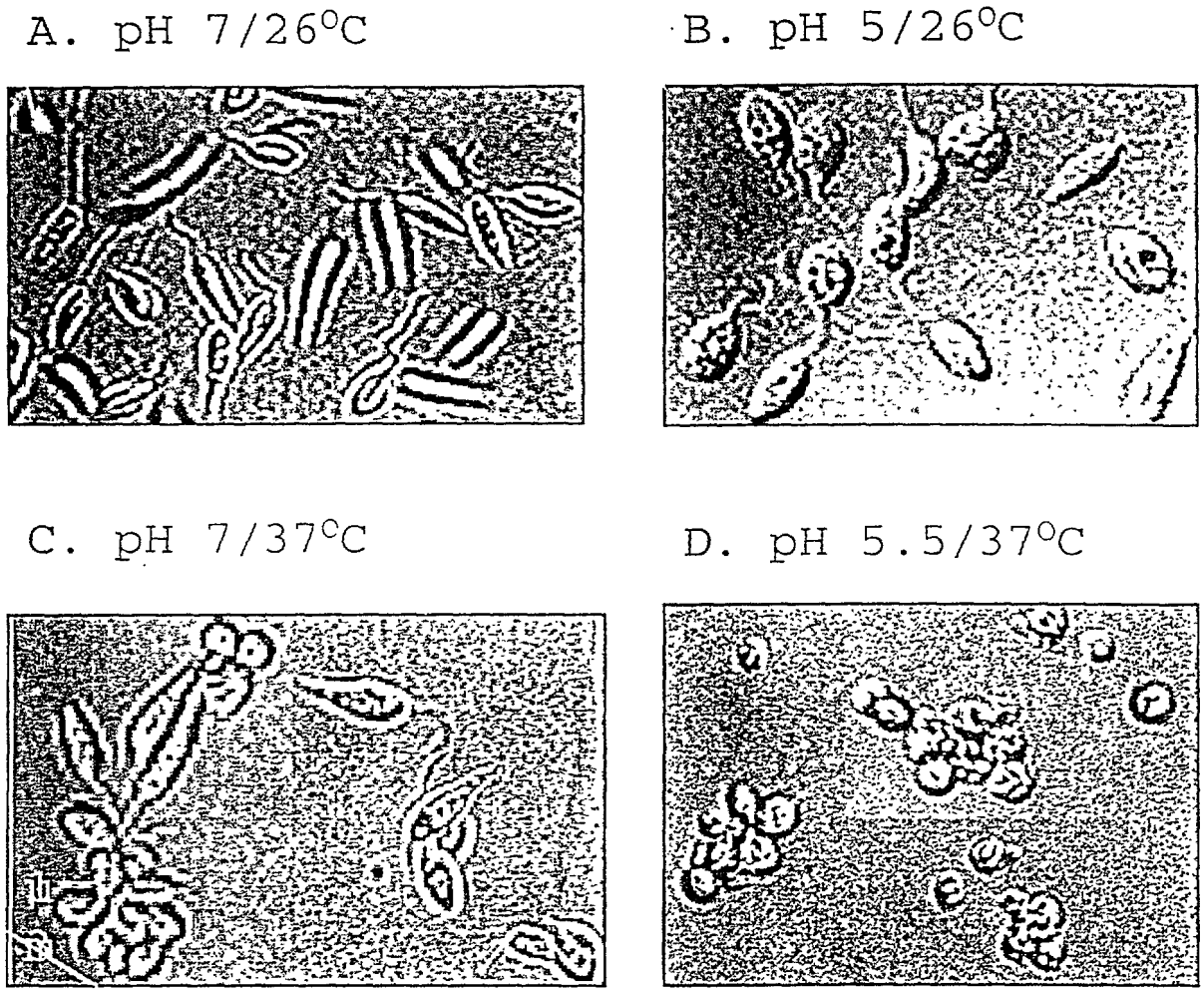


Figure 1. Nomarski micrographs of *in vitro* cultures of *L. donovani*. (A) Promastigotes at pH 7.4 at 26°C ; (B) "acidic" promastigotes at pH 5.5 at 26°C; (C) "heat adapted" promastigotes, i.e. parasites that have been grown for 24 hours at 37°C at pH 7.4; (D) *in vitro* amastigotes at pH 5.5 at 37°C.

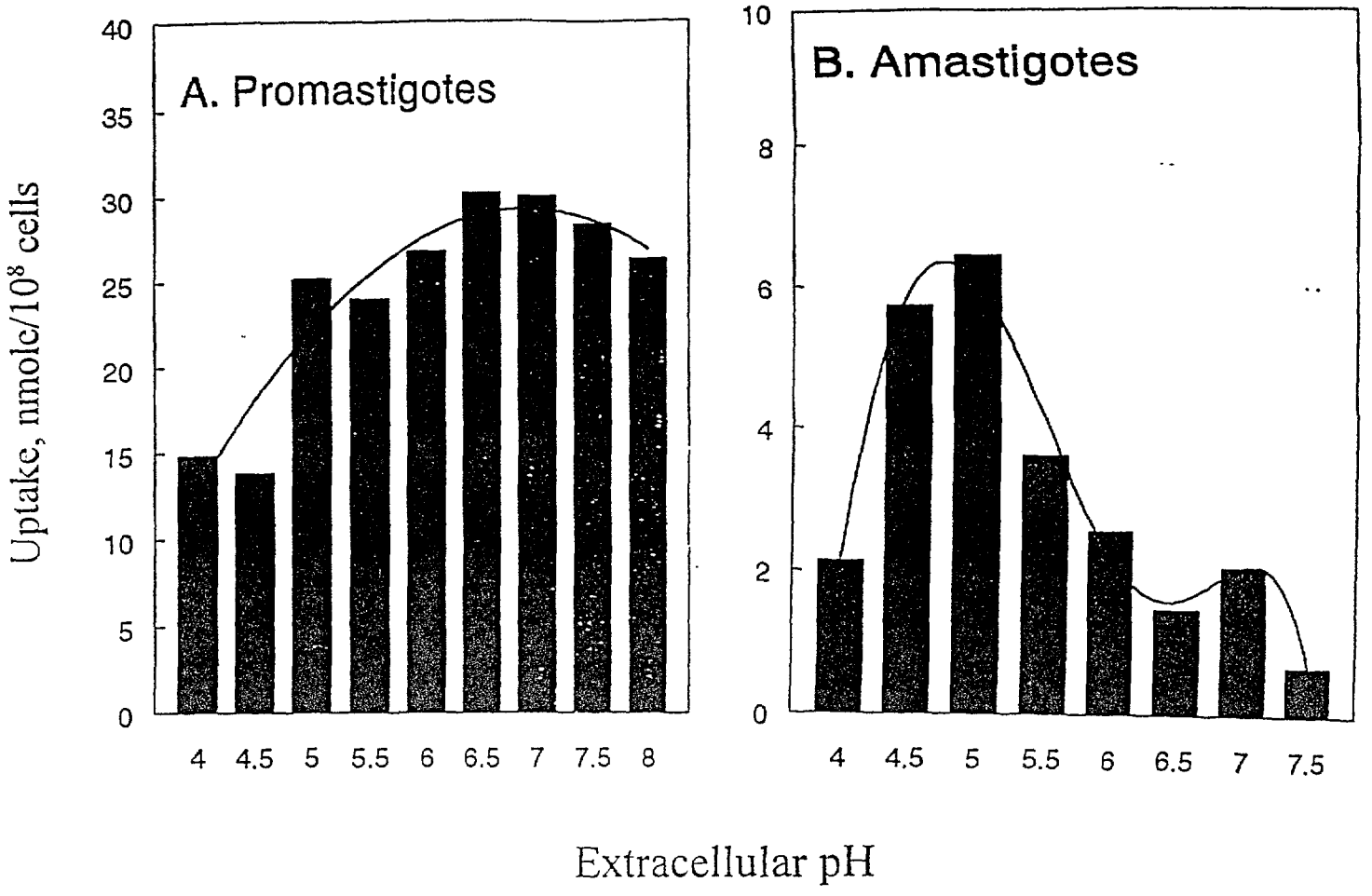


Figure 2. **pH response of proline transport in *L. donovani*.** Five minutes uptake as function of extracellular pH was measured in (A) mid log promastigotes and (B) *in vitro* amastigotes.

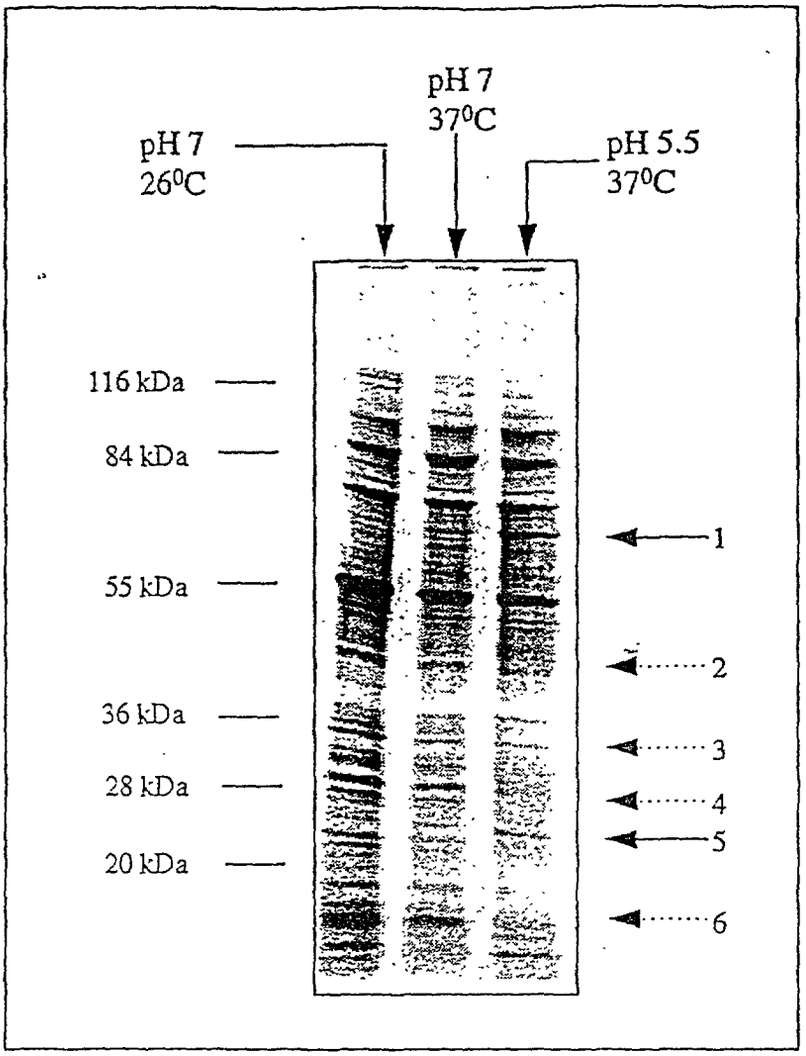
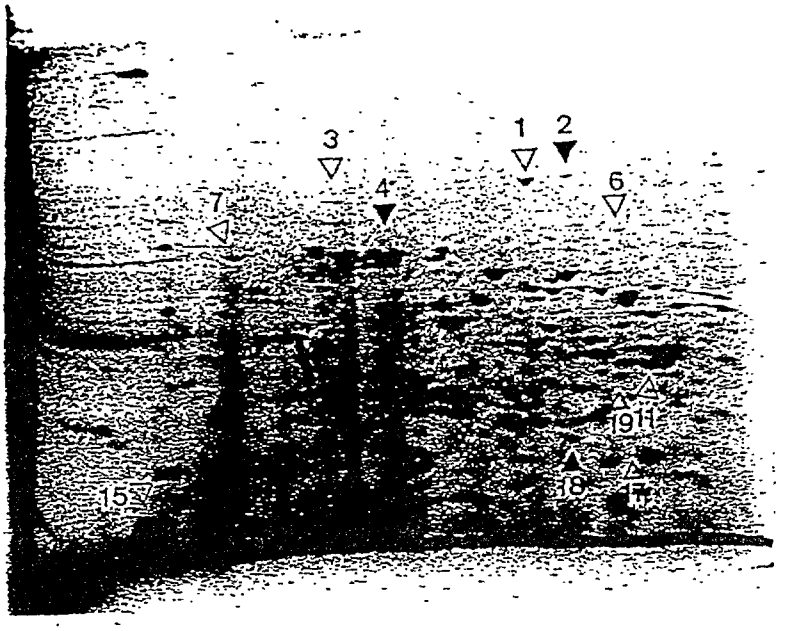


Figure 3. **Protein pattern of the various *L. donovani* cultures.** Cells from the cultures in Fig.1 were lysed and their proteins were separated on 9% SDS-PAGE. Each lane was loaded with 30 μg protein. Staining was carried out using Coomassie Brilliant Blue.



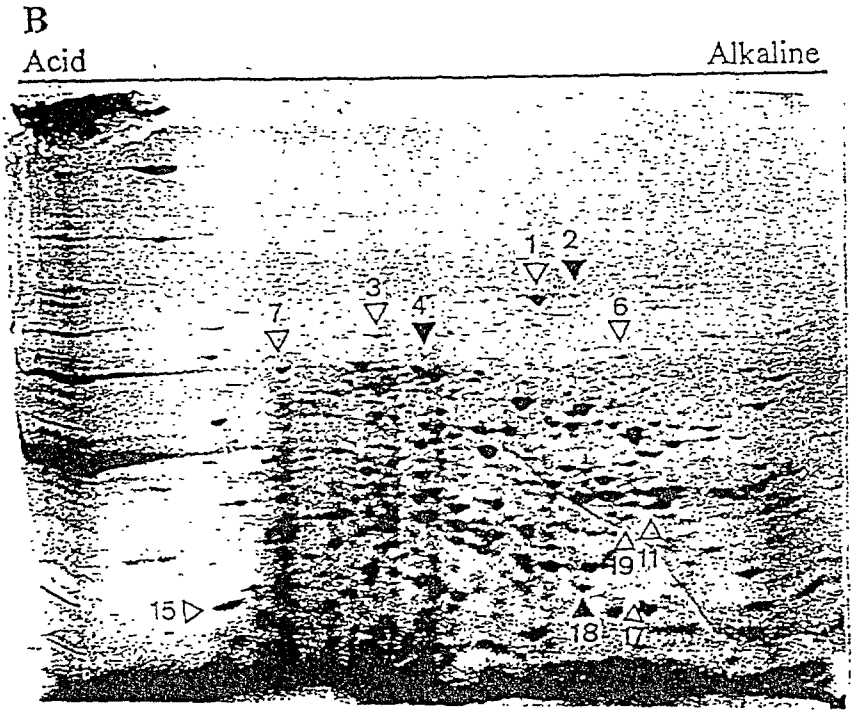
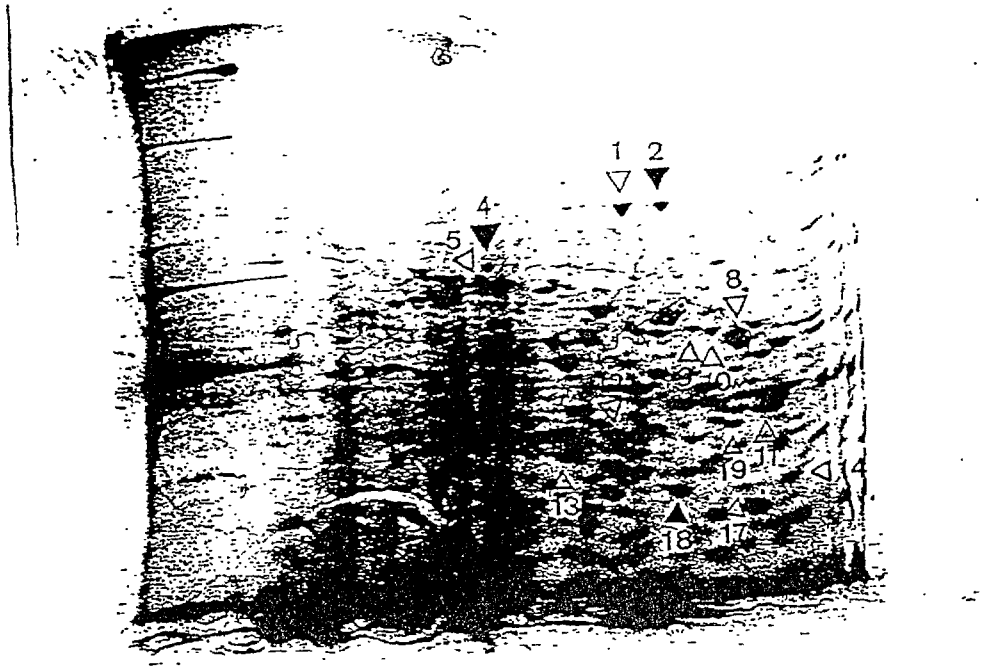


Figure 4. **Effect of pH on the rate of protein synthesis in *L. donovani* promastigotes.** Promastigotes were grown in medium 199 at 26^{0}C, harvested and resuspended to 5X10^7 cells/ml in 2 groups, one at pH 7 (A) and the other at pH 5 (B). After 1 hr of pre incubation, 20 μCi/ml of ^{35}S-methionine were added and incubation proceeded for additional 2 hours. Subsequently, cells were washed and separated on 2 dimensional PAGE. Black arrows indicate proteins whose synthesis increase at pH 5 and white arrows those whose synthesis decreased at the acidic pH.



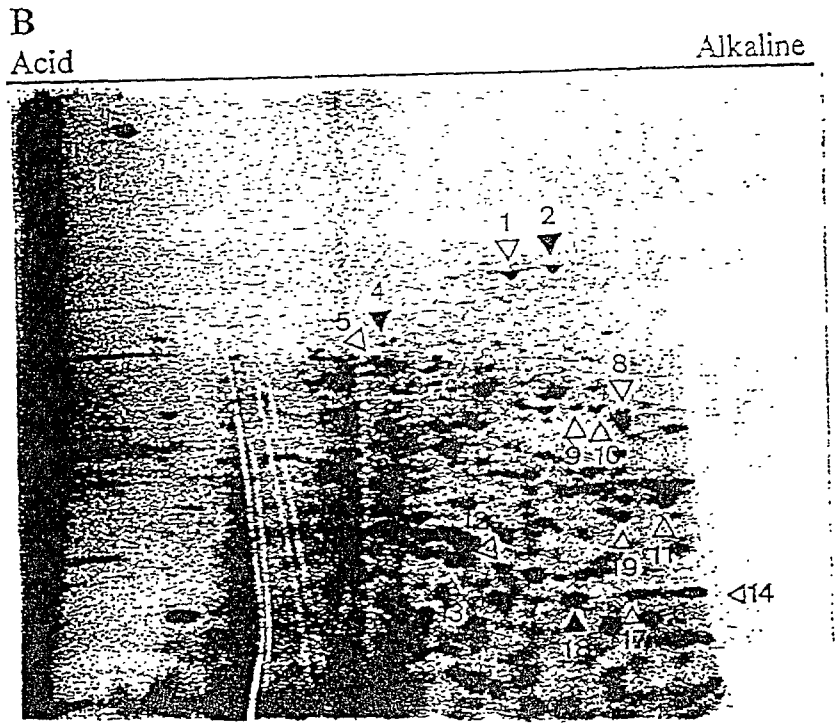


Figure 5. **Effect of pH on the rate of protein synthesis in *L. donovani* promastigotes that have been adapted to 37°C.** Promastigotes have been adapted to grow at 37°C in medium 199 that contained 25% fetal calf serum. Cultures were separated into two groups, one at pH 7 (A) and the other at pH 5. Both cultures were incubated for 1 hour at 37°C when 20 µCi/ml of ^{35}S-methionine was added. Incubation proceeded under the same condition for additional 2 hours. Subsequently cells were washed and treated as in fig. 4.

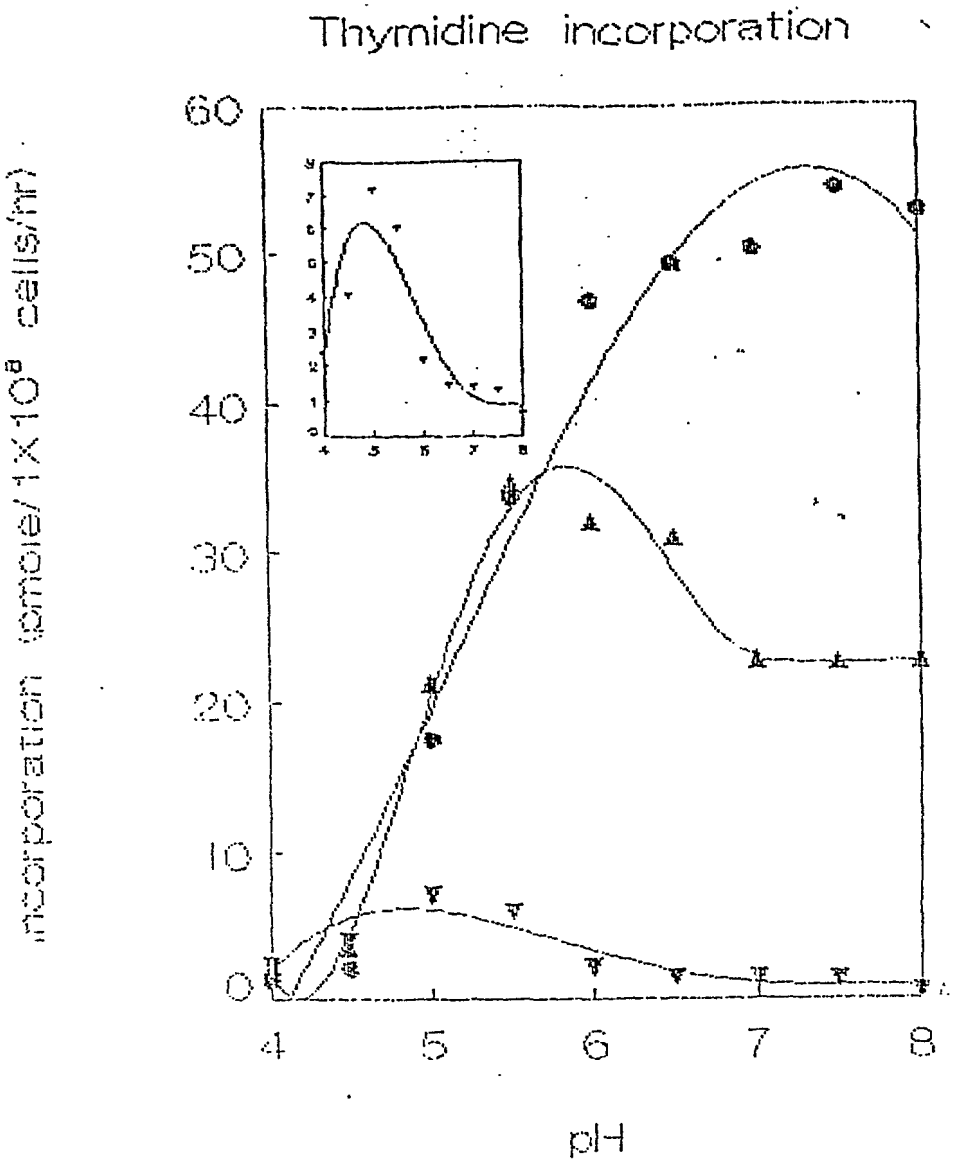


Figure 6. **pH response of thymidine incorporation in *in vitro L. donovani* amastigotes and promastigotes.** Mid log cultures of were harvested and washed twice in Earl's solutions at pH 7 for promastigotes (●) pH 5.5 for acidic grown parasites (○) and *in vitro* amastigotes (▼). Parasites were suspended to $5X10^7$ cells/ml, ^{3}H-thymidine was added and cells were then incubated at their appropriate conditions for 4 hours. Subsequently, 1 ml aliquots of cell suspensions were transferred to 5% ice-cold trichloroacetic acid , incubated on ice for 1 hour and then filtered through GF/C filter. Radioactivity was subsequently determined.

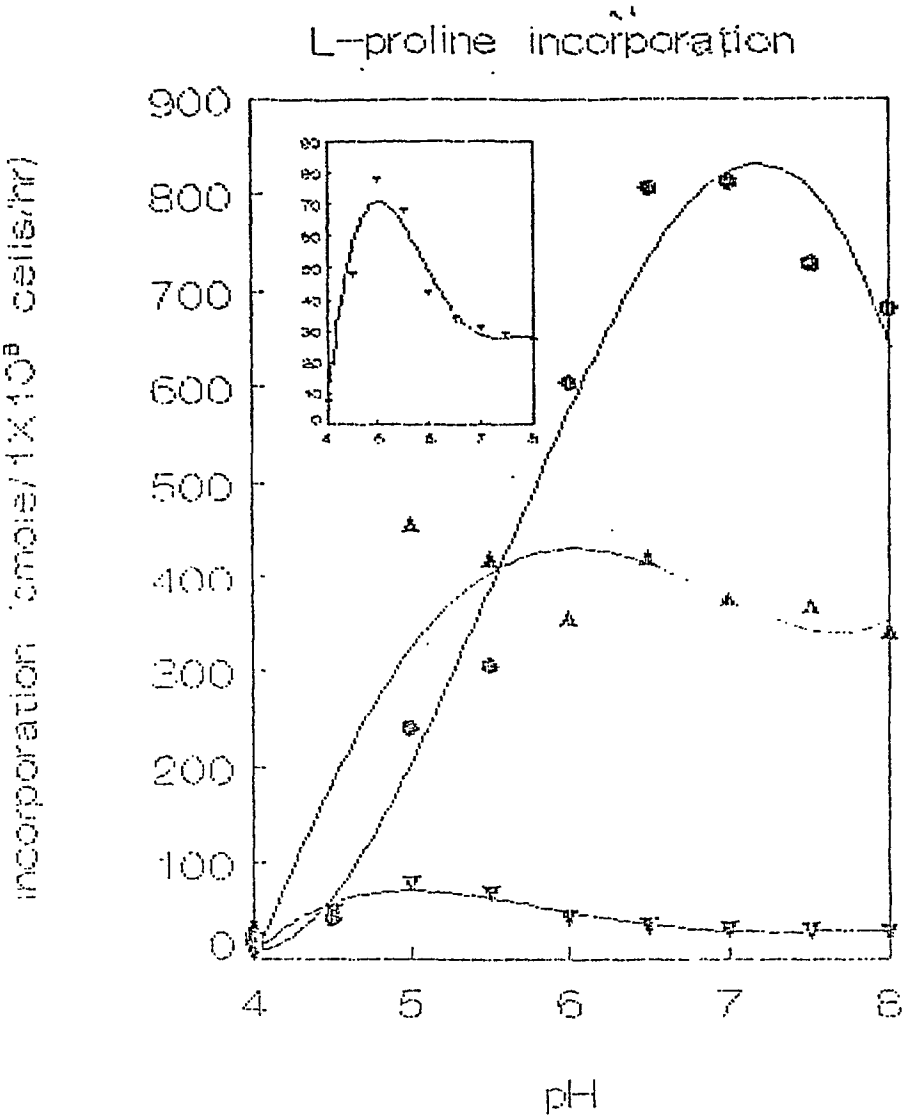
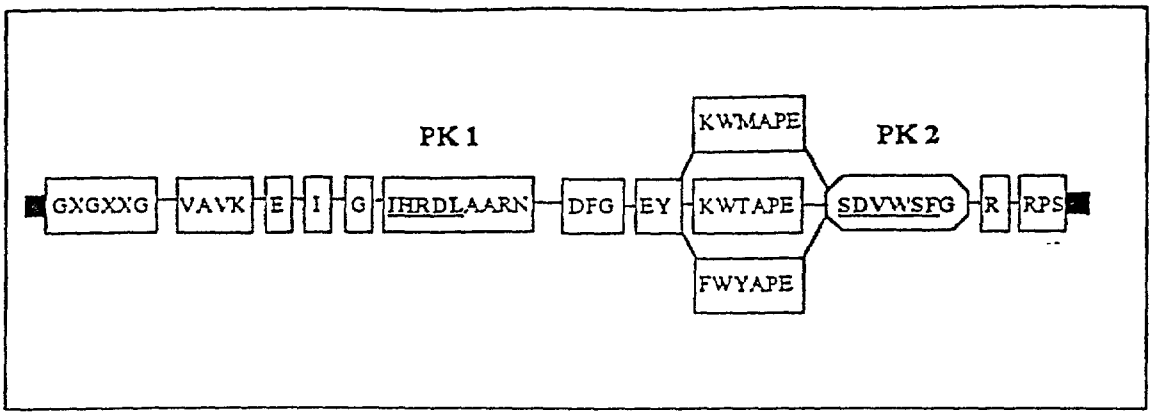


Figure 7. **pH response of proline incorporation in *in vitro L. donovani* amastigotes and promastigotes.** Cells were treated as in fig. 6 except that ^{3}H-proline was added to the cells for the metabolic labeling.



PK I-5'-CGGATT(C)CACCGC(G)GACCT-3'

PK II-5'-GGAATTGAAG(C)G(C)T(A)CCAG(C)ACGGTC-3'

Figure 8. The catalytic region of protein kinases. Conserved amino acids are indicated in boxes.

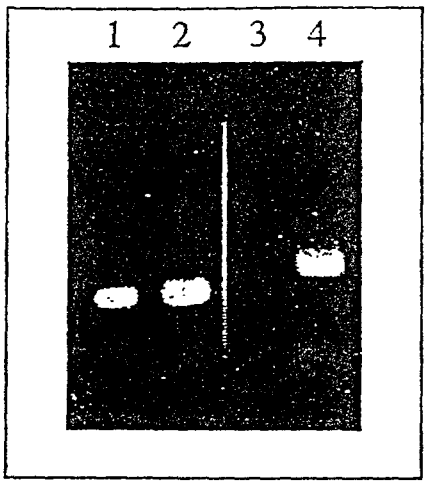


Figure 9. Amplification of *L. donovani* promastigotes genomic DNA with PKI and PKII primers.

```
A1-IHRDLKTSNVLLGEDGYVKVCDFGLGRLYREDQALTPAVVTLMYRAPELHF
A2-*******************************G*****T*************
A3-*******************************G*****T*************

A1-GVVDYSHKMDVWTF
A2-*A**********S*
A3-************C*


B- IHRDLKTSNIFVTSKNHLKLGDFGVCTNPVEPKRQGGEHERYPSLLRPEVC
   NSDPHVVERSDVESF

C- IHRDLNSDNLLLGENNVLKVCDFGLSXSRRRAVSTTTRYSSPXLGKAVLIX
   QAPELKERGXEGXSCDVWSF
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Figure 10. **The amino acid sequences of the PCR products amplified using *L. donovani* genomic DNA as template.**

```
A1-Ldp-   IHRDLKTSNVLLGEDGYVKVCDFGLGRLYRED-QALTPAVVTLMY
p58GTA-   L********L**SHA*IL**G****A*E*GSPLK*Y**V****W*
Spcdc2-   ******PQ*L*IDKE*NL*LA****A*SFGVPLRNY*HEI***W*
LmCRK1-   L*****PQ***ISREKEL*LG******SFAIPVRKFTNE****W*

A1-Ldp-   RAPELHFGVVDYSHKMDVWTF
p58GTA-   *****LL*AKE**TAV*M*SV    57%   homology
Spcdc2-   ****VLL*SRH**TGV*I*SV   44.6% homology
LmCRK1-   *P*DVLL*SMQ*GPPV***SV   41.5% homology
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Figure 11. **Sequence homology between A1 and the catalytic region of cdc 2-related protein kinases.**

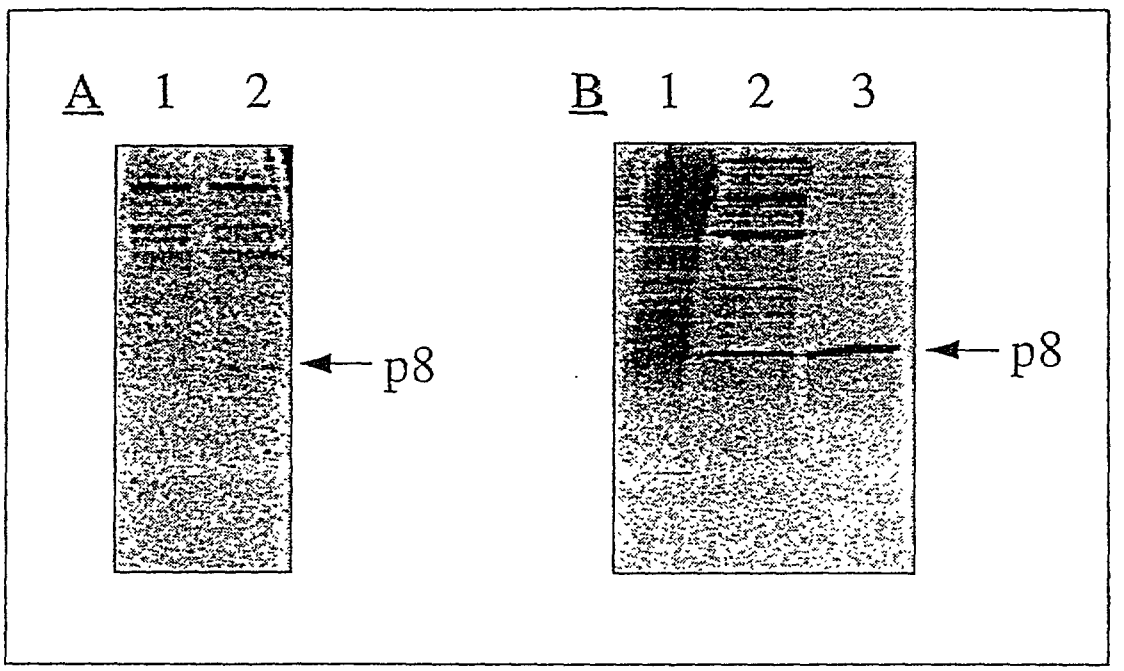

Figure 12. **Purification of recombinant p8.** A1 was subcloned in frame into pQE. IPTG-induced bacterial culture were lysed (1). Insoluble fraction (inclusion bodies) were solubilized using urea (2), loaded on nickel affinity column and eluted using imidazole (3).

C) Scientific impact of collaboration

Since the CO PI started his project few months after the Israeli PI, it is too early to evaluate the impact of collaboration. We plan on sending the antibodies as well as PCR probes to Peru so that the CO-PI group will clone the corresponding genes in the New World *Leishmania* species that they use. Also, both the Israeli and Peruan groups have established axenic cultures of amastigotes of both Old world (Israel, see section B.1) and New World (Peru, see section B.3.). This will enable us to complement each other and to ask similar question concerning the role of protein kinases in leishmanial differentiation.

D) Description of project impact

We feel that it is too early in the development of the project to report on any use of our results in either country. However, the use of axenic amastigotes culture is being used in growing number of laboratories. We believe that the paper that we are currently preparing on the subject will increase the interest of many scientists in the field of leishmaniasis.

E) Strengthening of Developing Country Institution

The Israeli PI's graduate student, Dr. Yehosua Saar visited the CO-PI laboratory last year. He gave a seminar and exchanged information with the group in Peru. Also, Dr. Saar gave the CO-PI's group information on how to perform the 2D gel analysis of protein phophorylation.

We plan that a graduate student from the CO-PI laboratory will come to the PI laboratory to help cloning protein kinase genes that are involved in *Leishmania* differentiation.

F) Future work

Now that we have on one hand DNA that encode for active regions of protein kinases and on the other hand *in vitro* amastigotes, we can look at mechanism of differentiation. The following will be the experiments we shall conduct during the next 12 months.

1. Cloning and sequencing and characterizing protein kinase genes in *L. donovani* using the DNA that have been amplified as described in section B.2.
2. Raising antiserum against p8 (section B.2). The antibodies will be exploited to identify its corresponding protein kinase in parasite cells. We shall also look at the stage regulation and expression of newly identified protein.
3. The axenic cultures of amastigotes and promastigotes will be used to further analyze the role of protein phosphorylation in leishmanial development.

As can be seen in this project, we follow our original objectives and believe that we will be able to accomplish most of these plans.

SECTION II

A) Budget

Budget summary is enclosed and signed by the Technion's authorities. The CO-PI's comments are in section IIB of his report. There is no change in the Israeli PI budget.

B) Collaboration and training

As stated in section E of this report and II.B of the CO-PI's report, Dr. Yehoshua Saar from the PI's laboratory visited in Lima. More detailed information is given in section II.B in the CO-PI's report.

The CO-PI plans on visiting in Israel at the beginning of 1997. The Israeli PI will also visit Lima during Spring of 1997. Also, a student from Peru will come to Haifa to conduct experiments.

(The CO-PI's future plans are described in section C of his paper).

Part II - Co - principal investigator

PROGRESS REPORT No 1

Covering Period: November 1995 - May 1996

Submitted to:

PRINCIPAL INVESTIGATOR: Dr. Dan Zilberstein
TECHNION INSTITUTE

"THE ROLE OF PROTEIN PHOSPHORILATION IN DIFFERENTIATION OF LEISHMANIA PARASITES"

CO-PRINCIPAL INVESTIGATOR: MARTIN LOPEZ
Grantee Institution: Universidad Peruana Cayetano Heredia

Principal Collaborators

CARLOS SANTA CRUZ, Research Associate
ROSA GARCIA, Research Associate
CECILIA CHOQUE, Technician
JUANA GOMEZ, Secretary

Project Number: USAID TA-MOU-94-C13-074

Project Duration: November 1995 - January 1999

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EXECUTIVE SUMMARY

The project aims to elucidate the role of protein phosphorilation in the development of *Leishmania* parasite, for this reason our preliminary work was devoted to obtain amastigotes like forms in order to study this process in all the parasite life stages.

We are working with three different species: *Leishmania peruviana, Leishmania braziliensis*, and *Leishmania guyanensis*. They're adapted to M199 medium, supplemented with 20% Foetal bovine serum. The three species are infective, and were previously characterized by Isoenzymes, molecular karyotyping, PCR and RAPD.

Currently, we can produce amastigotes by infection of human or mouse macrophage cell lines, U937 and J774 respectively, following purification through a Percoll gradient. Furthermore, amastigotes like forms would be obtained by incubation of the promastigotes to 34°C / pH 7.0 or pH 5.0

SECTION I

A) Research Objectives

Our knowledge of the differentiation mechanisms of the insect form of *Leishmania* (promastigote) to the intracellular form (amastigote) is relatively poor. As the main objectives of the project is to elucidate the role of protein phosphorilation in the development of this parasite, our preliminary work was devoted to obtain amastigotes like forms in order to study this process in all the parasite life stages.

The specific aims were:

1.- To adapt Leishmania isolates to medium 199.

2.- To produce amastigotes by infection of macrophages.

3.- To obtain Amastigotes-Like forms in culture.

B) Research Accomplishments

1.- <u>To adapt isolates to medium</u>.-

At the Instituto de Medicina Tropical "Alexander von Humboldt" we mantain the leishmanias at 24°C in a biphasic culture medium (rabitt blood agar/0.9% saline solution). In order to compare our results with the ones obtained at TECHNION, we adapted our leishmania isolates to Medium 199 suplemented with 20% foetal bovine serum.

Parasites mantained in culture were previously inoculated to hamsters (nose). One month later, amastigotes were obtained through aspiration of the lesion and the parasites were initially grown in biphasic medium and then passaged to M199-20%FBS. The animal infection was necessary in order to be sure that parasites to be used in the project are all infective.

It takes nearly 2 months for parasite adaptation to the new medium.

Parasites were all isolated in Peru and they were previously characterized by isoenzymes, PCR, RAPD and molecular karyotyping.

The following isolates are currently used:

Reference Number	Source	Locality	Species
MHOM/PE/90/HB22	Man[1]	Huancabamba	*Leishmania peruviana*
MHOM/PE/87/H166	Man[2]	Huancavelica	*Leishmania braziliensis*
PRN/PE/94/P52	Vector[3]	Ancash	*Leishmania guyanensis*

[1] Cutaneous lesion

[2] Mucosal Lesion

[3] *Lutzomyia peruensis*

2.- <u>To produce Amastigotes by infection of macrophages</u>.-

We have available the human macrophage cell line U937 and the mouse macrophage cell line J774.1. Both cell lines are susceptible to infection with the three leishmania isolates.

Macrophages are mantained in 50 ml Falcon tissue culture flasks containing M199 supplemented with 20%FBS, in an incubator at 37°C with 5% CO2.

Before infection, both parasites and macrophages are washed with 199 medium and their viability are tested by cell staining using a mixture solution of 5 mg/ml Fluorescein Diacetate, 2 mg/ml Ethidium Bromide, 0.5 mg/ml Orange Acridine, all in Hanks salts. Cells are observed under UV microscope. Viable cells have a green stained whereas death cells an orange color.

An standard protocol of infection is carried out at 34°C with a ratio of 20:1 leishmania/macrophage. Promastigotes are put in contact with the macrophages not more than two hours, then the parasites are washed out with temperated M199/20% FBS. The macrophages are then incubated for 3-4 days and evaluated for infection by fixation of the cells with 1% glutaraldehyde in M199 and stained with a mixture of May-Grünwald/Giemsa colorants. Infection results using the macrophage cell line J774.1 are shown in figure 1.

To obtain amastigotes, a passage of macrophages to a 250 ml tissue flask is done before infection. Macrophages at 70% confluence are infected with promastigotes and follow the same steps mentioned before. Cells are scraping from the bottom of the tissue culture flask using a rubber policeman and macrophages are lysed by repeated passage through a 27 gauge needle. Amastigotes are separated from cell debris on a discontinous 25/45/70% Percoll gradient.

3.- To obtain Amastigotes-Like forms in culture.-

One advantage of having an *in vitro* macrophage-free differentiation system of promastigotes to amastigotes is that we can follow changes on protein phosphorylation during the course of that process. In order to obtain amastigotes-like forms we growth all the *Leishmania* isolates at 34°C either pH 7.0 or acidic pH (5.0, using 10 mM Succinic Acid). As controls we mantain the parasites at 24°C either pH 7.0 or pH 5.0.

Figure 2 and 3 show promastigotes of *Leishmania peruviana*at 24°C/pH 7.0 and 34°C/pH5 respectively. Changes of temperature and pH gave as result round cells similar to intracellular amastigotes. Nevertheless, we're not able yet

to mantain viable those cells for more than 1 week. It seems similar to amastigotes-like reported in literature and we think that medium should be supplemented growth factors in order to obtain cell division.

To test the capability of round cells to continue their division inside a cell, they were used to infect mouse peritoneal macrophages. The result is shown in figure 4. The infectivity of these forms is similar to either promastigotes or intracelullar amastigotes (more than 90% infected macrophages).

C) Future Work

Currently, we have available promastigotes, amastigote-like forms and intracelullar amastigotes of three different *Leishmania* species from the New World to look for protein phosphorylation.

We are planning to look for autophosphorylation on intact cells and phosphorylation activity in crude extracts. We expect to find proteins related to differentiation or stage-specific. Selected proteins will be subjected to analysis of the phosphorylated aminoacid (Tyrosine, threonine, or serine).

We will perform experiments of infection in the presence of specific inhibitors or activators of Protein kinases.

On the other hand we will start the construction of full length cDNA libraries from *L. peruviana* (HB22).

Figure 1:

Macrophage J774 infected with *L.peruviana* (HB 22).

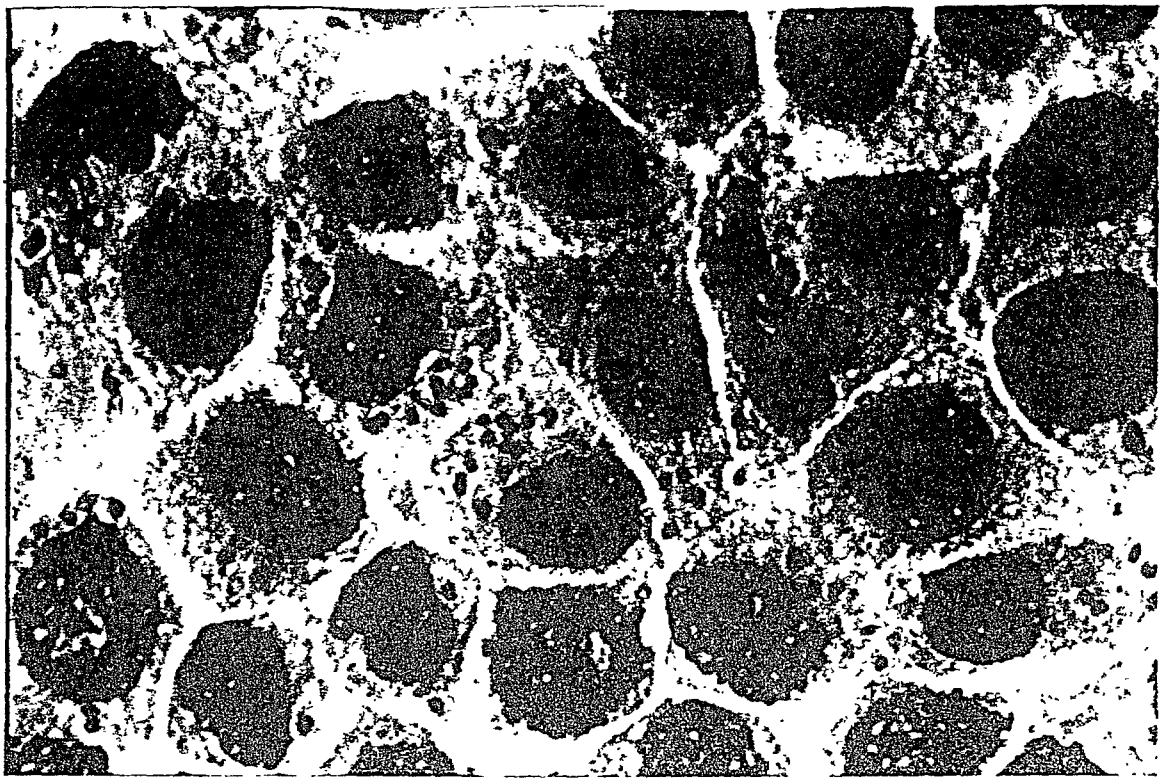
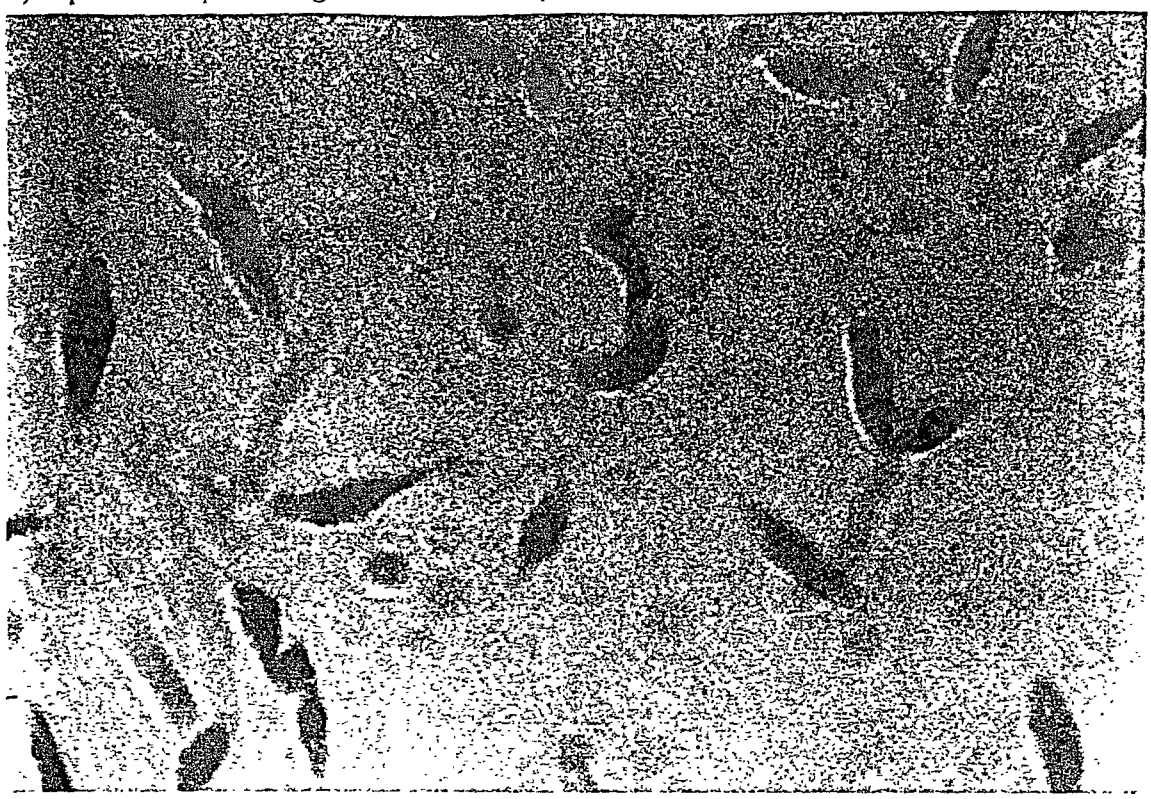


Figure 2:

a) L.peruviana promastigotes 24º C and pH 7



b.- The viability test of *L. peruviana* promastigotes using the fluorescein technique The viable cells are green stained whereas the death cells are orange

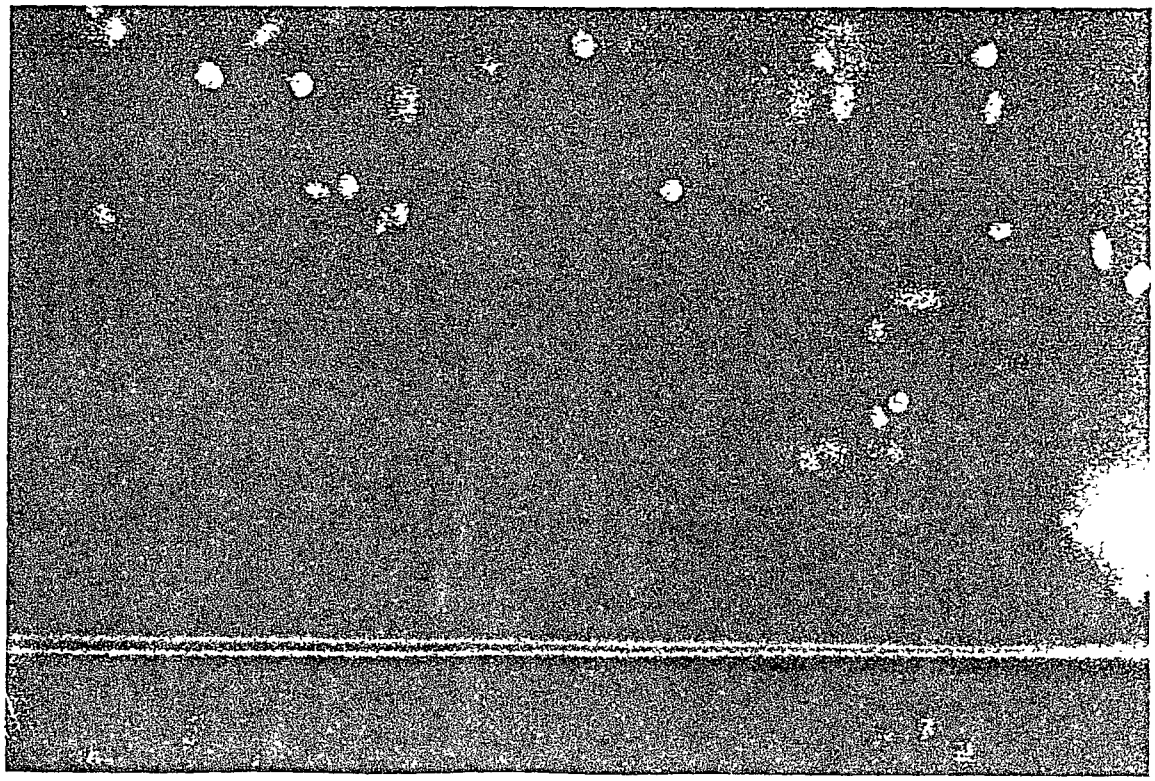
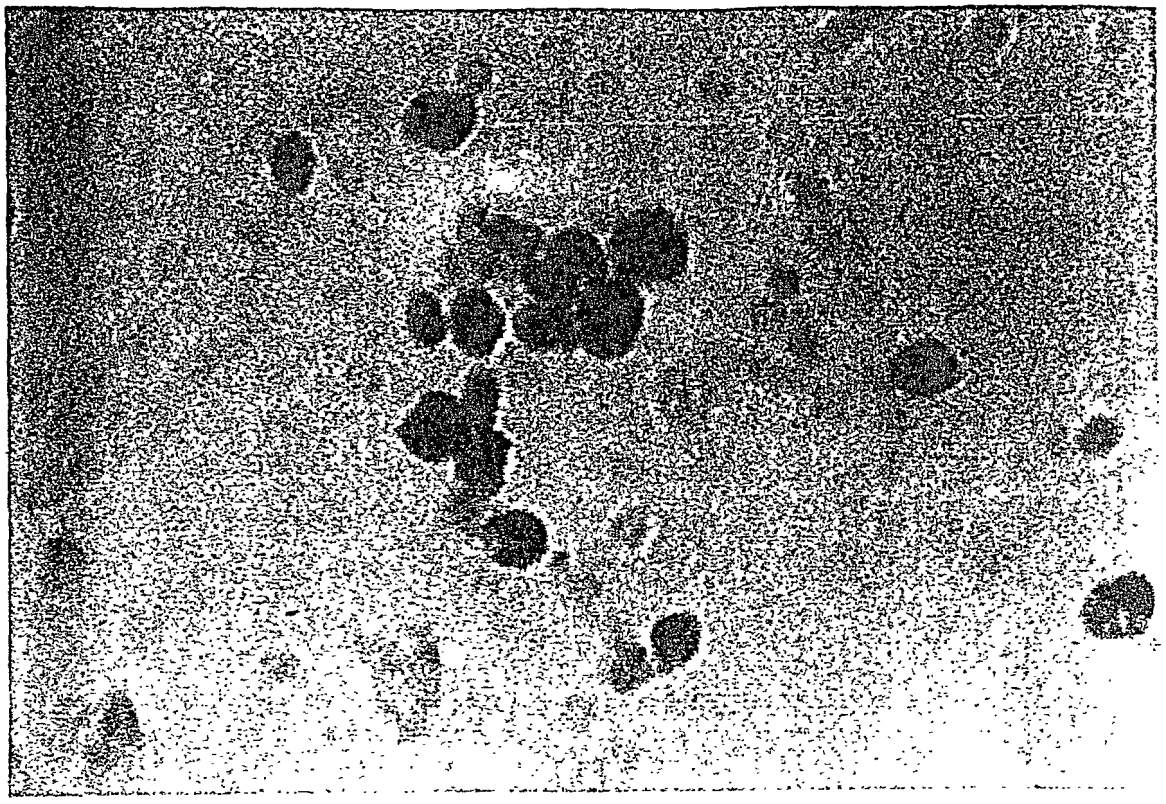


Figure 3:

a) "Amastigotes-Like" of *L.peruviana* induced by temperature and pH (34° C and pH 5).



b.- The viability test of "Amastigotes-Like" using the fluorescein technique The viable cells are green stained whereas the death cells are orange.

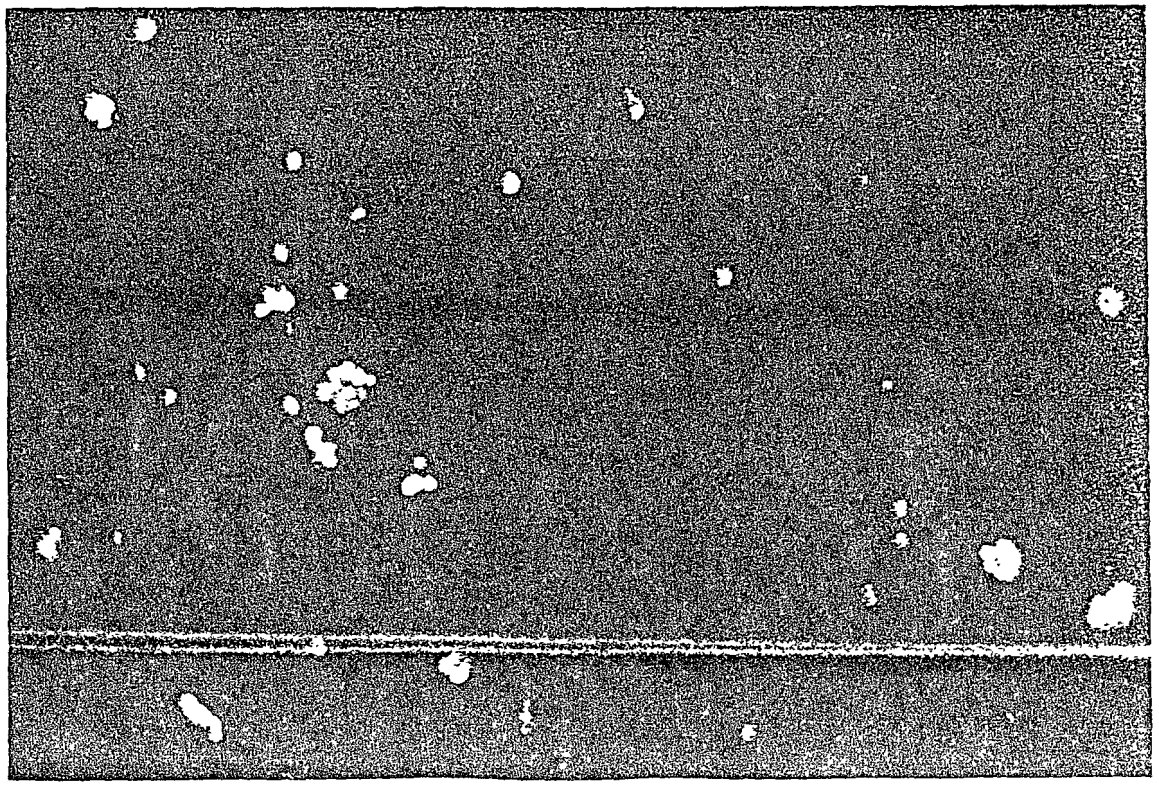
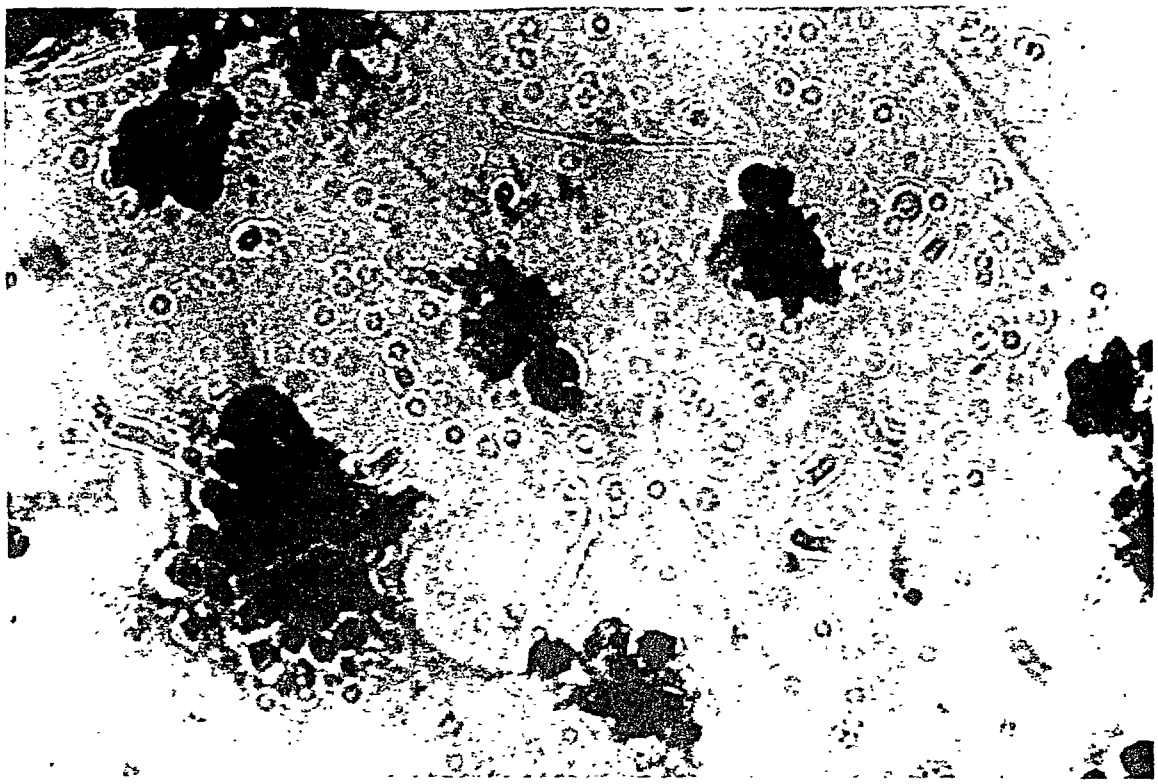


Figure 4:
a) Mouse peritoneal macrophage infected with "Amastigotes-Like" (34° C and pH 5). of *L.peruviana*



SECTION II

A) Budget

See attached budget balance signed by the UPCH authority. As you can observed there is an amount of money that must be reimbursed by USAID. Most of the first payment were derived to material, supplies and salaries. It is also enclosed the budget requested for the following period (June-December 1996).

B) Collaboration and Training

We received the visit of Dr. Yehoshua Saar from the TECHNION. The scientific exchange and discussions were very rewarding to our group. Dr. Saar gave a conference on Differentiation of *Leishmania donovani* promastigotes to amastigotes from the biochemical characterization point of view at Universidad Peruana Cayetano Heredia.

We expect that a member of our group will go to TECHNION for training on September 1996. The Co-Pi is also planning to visit Dr. Dan Zilberstein at the end of this year or January 1997. The latter will be discussed in a near future.