Serum deprivation leads to growth inhibition and protein tyrosine phosphorylation in *Entamoeba histolytica*

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Growth of *Entamoeba histolytica* in culture was found to be entirely dependent upon the presence of serum in the culture media. Very little protein phosphorylation was detected in growing *E. histolytica* cells by quantitative immunofluorescence and immunoblotting. Intense augumentation of antiphosphotyrosine immunofluorescence was observed after 1 h of serum deprivation. Antiphosphotyrosine immunofluorescence levels increased moderately between 1 h and 1.5 h of serum deprivation, after which no further increase was observed till 4 h serum deprivation. At 1 h of serum deprivation, a very high tyrosine kinase activity was detected. Sodium orthovanadate, a potent tyrosine phosphatase inhibitor, increased tyrosine phosphorylation of a 38 kDa protein after 1 h of incubation and also inhibited the growth of *E. histolytica* in culture. Our data demonstrated that protein tyrosine phosphorylation was associated with growth inhibition of *E. histolytica*.

ENTAMOEBA histolytica, a parasitic protozoan, causes amoebic dysentery and amoebic liver abcess in populations inhabiting the tropical regions of the world. The organism gains entrance into the host's body in the non-dividing dormant form called the cyst. The cyst transforms into the trophozoites in the host intestine and rapidly multiplies in number. When faced with environmental stress, such as nutrient deprivation, change in temperature, pH, etc., the trophozoites change into cysts and can remain dormant for a long period[1]. Very little information is available about the mode of cyst formation in *E. histolytica*. Unfavourable conditions are known to change trophozoites into cysts but the underlying biochemical mechanisms have received little attention from researchers in recent years. In this study we have looked into the changes in protein phosphorylation events after serum withdrawal in *E. histolytica* in an attempt to correlate protein phosphorylation events with growth factor deprivation in *E. histolytica*.

We found that in growing control cells (48 h log phase culture), very little tyrosine phosphorylation could be detected by immunofluorescence. Serum deprivation for 1 h resulted in significant enhancements ($P < 0.001$; 7 fold) in antiphosphotyrosine immunofluorescence. When serum deprivation was prolonged further (1.5 h), moderate enhancements ($P < 0.001$; 10-fold over control) in antiphosphotyrosine immunofluorescence were observed over 1 h serum deprivation levels. No further increase was observed between 2 and 4 h of serum deprivation but the antiphosphotyrosine immunofluorescence levels remained steady between 1.5 and 4 h (Figure 1). Our observation clearly showed that growing cells had low levels of protein tyrosine phosphorylation and after serum deprivation protein tyrosine phosphorylation levels increased dramatically and remained higher for the entire period of observation (4 h serum deprivation). Results obtained with the immunofluorescence studies were in complete agreement with the findings of the immunoblotting experiments (Figure 2). Western blots of control cell lysates (Figure 2 *a*, lane 2) when probed with the anti-phosphotyrosine antibody, showed no detectable tyrosine phosphorylation. In contrast, blots containing lysates from cells after 1 h of serum deprivation demonstrated two prominently tyrosine phosphorylated proteins (38 and 42 kDa; Figure 2 *a*, lane 1). When serum deprivation of the cells was conducted in the presence of 25 μM Genistein, a potent tyrosine kinase inhibitor[2], almost no tyrosine phosphorylation was detectable on the immunoblot, demonstrating the specificity of the antibody for tyrosine phosphorylated proteins (Figure 2 *b*, lane 1).

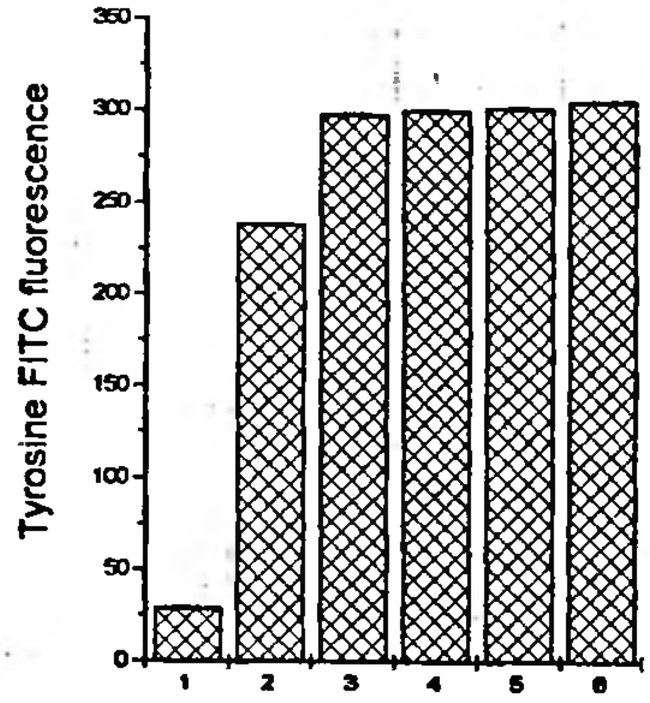

Figure 1. Antiphosphotyrosine immunofluorescence levels in control (48 h) and serum-deprived cells. Data are presented as mean ± SD. The SD bars are not visible in cases with small SD values. 1, Growing control cells (48 h); Cells deprived of serum for 2, 1 h; 3, 1.5 h; 4, 2 h; 5, 3 h and 6, 4 h. Cells were harvested from log-phase (48 h) culture (growing control), and after 1–4 h serum deprivation. Harvested cells were processed for immunofluorescence studies. After fixation in 90% ethanol in PBS (30 min; 4°C) and subsequent washing in PBS, the cells were incubated with the primary anti-phosphotyrosine antibody (mouse; clone PT66; Sigma) in 0.5% BSA (room temperature; 60 min). This step was followed by washings in PBS and incubation with the secondary antibody in 0.5% BSA (anti-mouse rabbit IgG-FITC conjugate, Bangalore Genei). Cells were then washed twice in PBS and fixed in 1% paraformaldehyde. The cell suspension was examined in a Hitachi F-4500 Spectrofluorimeter (excitation 495 nm and emission 525 nm). One negative control was measured omitting the primary antibody step and another negative control was measured by the addition of a step before the FITC-conjugated secondary antibody in which the cell suspensions were incubated with an unconjugated rabbit antimouse IgG. Both negative controls showed fluorescence values of < 5% of the peak fluorescence values.

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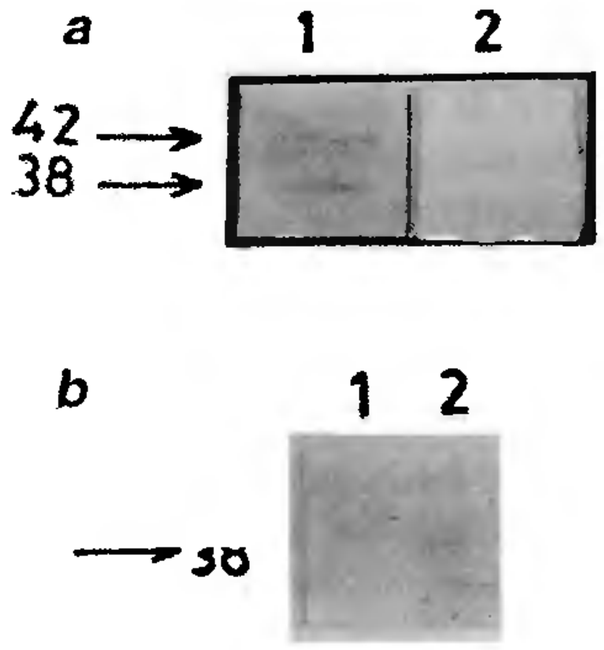

Figure 2. Immunoblot of *E. histolytica* cell extracts with anti-phosphotyrosine antibody. *a*, Lane 1, Cells were serum-deprived for 1 h; Lane 2, Control cells (log phase, 48 h culture); *b*, Lane 1, Cells (48 h culture) were serum deprived for 1 h in the presence of 25 μM Genistein; Lane 2, Cells (48 h culture) were incubated with 25 μM sodium orthovanadate in serum containing culture medium for 1 h. Proteins from *E. histolytica* cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The blots were probed by anti-phosphotyrosine antibody (mouse; clone PT66, Sigma; PY20, Calbiochem) and alkaline phosphatase colour reaction was carried out for the identification of bands. Similar amounts of protein were loaded in each lane.

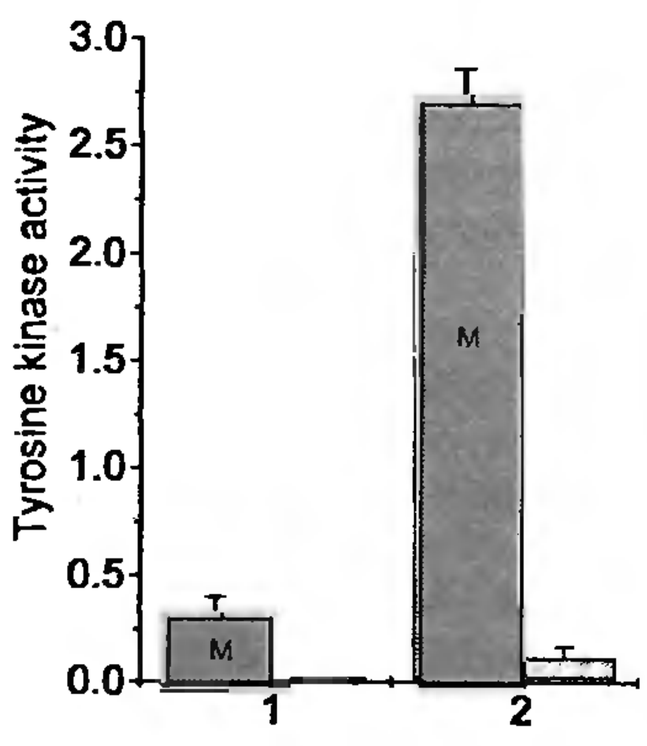

Figure 3. Tyrosine kinase activity in control (48 h) and serum deprived (1 h) cells. Data are presented as mean ± SD. 1, Growing control cells (48 h); 2, Cells serum deprived for 1 h . The deep grey column represents the membrane fraction and the light gray column represents the cytosolic fraction. Tyrosine kinase activity is presented as cpm ^{32}P incorporated/μg protein. Tyrosine kinase was assayed in the membrane protein fractions of *E. histolytica* by using a peptide (EGPWLEEEEEA[Y]GWMDF) from Pierce (USA) as kinase substrate. The assay mixture contained 10 μCi γ-^{32}P ATP (sp. act. 3000 Ci mmol^{-1}), 2.5 mM cold ATP, in buffer (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 30 μM sodium orthovanadate, 5 mM sodium fluoride). The reaction was allowed to proceed for 45 min at 37°C. All samples were assayed in duplicate. The mixture was centrifuged in phosphocellulose units (Pierce) and washed twice with 75 mM H_3PO_4. The level of incorporation of radioactivity was determined by scintillation counting. A blank was measured in the absence of cell extracts and absence of substrate for all samples and this value was routinely subtracted from the enzyme assay data. On an average, the blank value was not more than 5% of the assay values.

Table 1. Effect of serum and sodium orthovanadate on the growth of *E. histolytica* in culture

Percentage of serum	Cell number (% of control)
0	7.14 ± 0.014
1	14.29 ± 0.014
3	37.75 ± 0.014
5	56.12 ± 0.038
7	76.53 ± 0.025
10	100.00 ± 0.063
10 + 15 μM sodium orthovanadate	7.27 ± 0.01
10 + 2 μM sodium orthovanadate	0.00 ± 0.00

Data are represented as the mean ± standard deviation. Growth of *E. histolytica* in 10% serum was taken as 100%. Cells were harvested after 48 h of growth.

Low levels of tyrosine kinase activity were detected in the membrane fractions of control (48 h log phase) cells (Figure 3). In the cytosolic fractions of these cells, protein tyrosine kinase activity was barely detectable. However, after 1 h of serum deprivation, protein tyrosine kinase activity was found to be significantly increased ($P < 0.001$; 10 fold) in the membrane fractions. In the cytosolic fractions, the protein tyrosine kinase activity also increased slightly but was still 10-fold lower than the membrane associated tyrosine kinase activity.

Table 1 shows that serum is essential for the growth of *E. histolytica*. In the absence of serum, growth of *E. histolytica* in culture was completely inhibited. With increasing concentration of serum (1–10%), growth of *E. histolytica* in culture was directly correlated to the concentration of serum in the medium. A definite requirement of serum has been demonstrated for *E. histolytica* cultures by other investigators[3,4].

Sodium orthovanadate, a potent inhibitor of tyrosine phosphatase[5], potentiates growth factor action in mammalian cells[6,7]. We observed that sodium orthovanadate treatment for 48 h in culture resulted in drastic inhibition of growth (Table 1). Also, sodium orthovanadate (25 μM) treatment of log phase cells in the presence of serum for 1 h resulted in the tyrosine phosphorylation of a 38 kDa protein in the cell extracts (Figure 2 *b*, lane 2).

We observed that serum was necessary for the growth of *E. histolytica* and serum withdrawal from the culture medium resulted in growth inhibition of this parasite. As serum withdrawal was also accompanied by increased protein tyrosine phosphorylation we conclude that tyrosine phosphorylation of certain proteins in *E. histolytica* may accompany growth inhibition while in growing cells protein tyrosine phosphorylation levels are very low. In contrast, growing mammalian cells show more tyrosine phosphorylated proteins than serum-deprived quiescent cells[8]. Our data emphasize the possibility that important

differences could exist in the relay of a growth signal between this parasite and its mammalian host.

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In vitro propagation of a Himalayan pine *P. wallichiana* A. B. Jacks

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Four different plant tissue culture media containing varying levels of growth hormones were studied to determine the nutritional and hormonal requirements for induction and subsequent elongation of shoot buds and rooting. Germinating embryos as explants placed in an inverted position in LP medium containing BAP produced adventitious shoot buds. Activated charcoal treatment of the embryos after being exposed to BAP was necessary for shoot proliferation and elongation. Elongated shoot buds were passed to rooting media. While using this technique for micropropagation of other pines, it would be necessary to optimize the age of the explant and the duration of exposure in shoot induction medium.

PINUS wallichiana (Bhutan pine or Blue pine syn. *Pinus excelsa* Wall.) is native to temperate Himalayas at elevations of 6000 to 12,000 ft. It extends westward to Afghanistan and eastward to Bhutan[1]. In western Nepal it is found up to an elevation of 14,000 ft (ref. 2). Needles of this plant are present in fascicles of five. Next to *Cedrus deodara* this is the most important coniferous timber tree of the Himalayas. It is also used for production of turpentine and tar. *P. wallichina* and *P. roxburghii* are the two major pine trees of Nepal; *P. patula* being the only minor species.

Application of *in vitro* methods to clone select genotypes provides an opportunity to realize large genetic gains more rapidly than is possible using conventional tree improvement methods[3–5]. Pine seeds mature twelve months after fertilization; two years intervene between initiation of ovules and formation of seeds. Out of about 90 *Pinus* species, around fifteen have been amenable to *in vitro* micropropagation. The monsoon belt of outer Himalayas suffers with a serious problem of desertification due to erosion of top soil and efforts to regenerate *P. wallichiana* by tissue culture methods can be extremely useful. Pine forests of this region have been reported to suffer from infection by a fungus *Cronartium himalayense* causing stem rust[6] and availability of a tissue culture method would make this species amenable to genetic improvement. Though not fast growing, pine species are fairly drought-resistant. Use of tissue cultured planting material can help in large-scale afforestation programmes.

The present paper describes the nutritional and hormonal requirements for induction and subsequent elongation of shoot buds for rooting in *Pinus wallichiana*. Development of a micropropagation protocol will be helpful as this species has been mainly used for breeding against blister rust (*Cronartium ribicola* Fisch.). The literature provides no information on the tissue culture of this species.

Mature seeds of *P. wallichiana* were obtained from Forest Development Project, Hattishar, Kathmandu and stored at 4°C. Seeds were washed in liquid detergent for 15 min and rinsed thoroughly in running tap water. Seeds that floated in water were discarded while the remaining seeds were scarified at the micropylar end and kept at 4–8°C for 24 h barely submerged in a little of water. Seeds were sterilized with 20% clorox containing a drop of Tween 80 for 15 min and rinsed three times with sterile distilled water. Before placing megagametophytes on agar plates for germination in the dark at 27°C, testa and operculum of the seeds were removed asceptically. After 1–7 days of growth, cotyledons were dissected out from megagametophytes and used as explant to determine the optimal growth stage and various media compositions. However, in subsequent experiments where whole embryos were used as explant, embryos were excised from the megagametophytes germinated for 5 to 7 days in agar plates.

Four different media, Murashige and Skoog (MS)[7], Schenk and Hilderbrandt (SH)[8], Gresshoff and Doy (GD)[9], and Quoirin and Lepoivre medium (LP) modified by Aitken-Christie and Thorpe (modified LP)[10] were used in

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