STUDIES ON GROWTH

Dictyostelium cells in Ax2 media show a typical sigmoid growth curve with distinct lag, log and stationary phase with a generation time of ~12 hrs. Of different phases of growth, log phase cells were selected for all our studies due to its optimal responsiveness to the drug treatment. In our studies ISOPROTERENOL (Iso) and ALUMINUM FLUORIDE (AlF$_4^-$) were used as modulators of adenylate cyclase. Various parameter under which the physiological aspects of treated cells of Dictyostelium discoideum studied were,

(i) Growth and survival
(ii) Colony morphology
(iii) Folic acid chemotaxis and folate deaminase assay
(iv) Endocytotic activity
(v) Macromolecular syntheses
(vi) Cytoskeletal proteins

1 SURVIVAL AND GROWTH OF TREATED DICTYOSTELIUM CELLS IN AXENIC MEDIUM

1.1 SURVIVAL

Log phase cells (5 x 10$^6$ cells/ml) were treated with Iso. At concentration of 200 and 400 μM Iso showed negligible or no cell lethality. AlF$_4^-$ at 200 μM and 400 μM also showed negligible cell lethality as reveled by dye exclusion test.

1.2 GROWTH PROFILE STUDIES

Dictyostelium cells were treated with different enantiomers (+, – and +/-) and different concentration (100 μM – 400 μM) of Iso and 200 μM, 400 μM of AlF$_4^-$ for 30 min. The growing Dictyostelium cells in axenic medium showed a typical sigmoid curve with generation time of 10-12 hrs. In the control, Iso and AlF$_4^-$ treated cells showed similar lag phase of 18-20 hrs followed by log phase till 72 hrs and the stationary phase after 72 hrs. After brief period of stationary phase the cells enter into the senescence phase where the cells begin to lyse leading to lesser cell density.
1.3 Growth profile of Isoproterenol treated cells

Mid log phase cells of Dictyostelium were treated with Iso for 30 min and were resuspended in fresh axenic medium and their growth was monitored at regular interval of time. Our studies showed that maximum mitogenic stimulation in case of Iso treated Dictyostelium cells were achieved after 48 hrs of growth in axenic medium. (+) Iso at 100 μM, 200 μM, and 400 μM showed 27%, 42%, and 17% stimulation respectively at 48 hrs (fig. 3a). (-) Iso at 100 μM, 200 μM, and 400 μM shows a stimulation of 10%, 28%, and 12% at 48 hrs respectively (fig. 3b). 200 μM (+) Iso showed highest mitogenic stimulation of 42% after 48 hrs of growth as compared to the control cells (table I). Equimolar mixture of – and + type of Iso at 100 μM and 200 μM showed 18% and 14 % stimulation respectively at 48 hrs (fig. 3d). The mitogenic stimulatory effect of Iso on Dictyostelium cells is concentration and enantiomers dependent and in order of 200 μM > 400 μM > 100 μM and (+) Iso > (-) Iso > (+/-) Iso respectively (fig. 3c, table I). Since 200 μM (+) Iso treated Dictyostelium cells showed maximum mitogenic stimulation as, compared to either 400 μM or 100 μM Iso, it was selected as our operational dose for the rest of the studies.

1.4 Effect of propranolol

To check the specificity of mitogenic stimulation in Iso treated Dictyostelium cells its antagonist 200 μM propranolol (Pro) was used. The Iso stimulated cells were challenged with Pro, which is its specific antagonist. In pretreatment, the Dictyostelium cells were first treated by Iso for 30 min followed by Pro for 30 min, in post-treatment the order of treatment was reversed and in simultaneous treatment the cells were treated with Iso and Pro simultaneously. It was found that Pro was able to bring down the stimulatory effect of Iso to control level depending on the order of treatment (fig. 3e). It was found that the post-treatment with Pro effectively reduced the stimulatory effect of the Iso to the control level as compared to the pre and simultaneous treatment (fig. 3f).
Fig. 3 Growth curve of *Dictyostelium* cells treated with different isomers of isoproterenol for 30 min. a) + Iso, b) - Iso
Fig. 3  Growth curve of *Dictyostelium* cells treated with isoproterenol for 30 min. c) – and + Iso, d) ± Iso
Fig. 3 Growth curve of *Dictyostelium* cells treated with isoproterenol and propranolol for 30 min. 
e) Iso and pre, post, simultaneous Pro, f) Iso and Pro
Table I: Percentage stimulation or inhibition of Iso treated *Dictyostelium* cells as compared to control cells.

<table>
<thead>
<tr>
<th>Conc. (µM)</th>
<th>Time  (hrs)</th>
<th>ISOPROTERENOL (% stimulation/inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(+)</td>
</tr>
<tr>
<td>100</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>16</td>
</tr>
<tr>
<td>200</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>35</td>
</tr>
<tr>
<td>400</td>
<td>24</td>
<td>6</td>
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<td></td>
<td>48</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>22</td>
</tr>
</tbody>
</table>

"-" Sign indicated decrease in cell growth as compared to the control *Dictyostelium* cells. N.D. – not done

1.5 Growth profile of ALUMINUM FLUORIDE (AlF₄⁻) treated cells

*Dictyostelium* cells were treated with different concentration (200 µM and 400 µM) of AlF₄⁻ for 30 min and were resuspended in fresh axenic medium. 200 µM and 400 µM AlF₄⁻ showed maximum stimulation of 11% and 22% at 48 hrs respectively as compared to the control cells (fig. 4a, Table II). As 400 µM AlF₄⁻ treated *Dictyostelium* cells showed maximum growth stimulation as compared to the 200 µM AlF₄⁻, 400 µM AlF₄⁻ was chosen as our operational dose for the rest of our studies. It was found that the mitogenic stimulatory effect of AlF₄⁻ is lower as compared to Iso (fig. 4b) and is concentration dependent.
Fig. 4  A comparative studies on mitogenic stimulation of Dictyostelium cells treated with either different concentration of aluminum fluoride (AlF₄⁻) or Iso. a) AlF₄⁻  b) AlF₄⁻ or Iso.
Table II: Percentage stimulation or inhibition of AlF₄⁻ treated Dictyostelium cells as compared to control cells.

<table>
<thead>
<tr>
<th>Conc. (µM)</th>
<th>Time (hrs)</th>
<th>ALUMINUM FLUORIDE (AlF₄⁻) (% stimulation/inhibition of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>24</td>
<td>-2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
</tr>
<tr>
<td>400</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>14</td>
</tr>
</tbody>
</table>

"-" Sign indicated decrease in cell growth as compared to the control Dictyostelium cells.

1.6 Effect of aminophylline
In another set of experiment aminophylline an inhibitor of phosphodiesterase, inhibition of phosphodiesterase leads to increase in concentration of cytosolic cAMP which feedback inhibits adenylate cyclase. Aminophylline was used to check its effect on the growth of Dictyostelium cells. Aminophylline affects the growth of Dictyostelium cells in dose dependent manner (fig. 5). At 200 and 100 µM aminophylline showed growth inhibition of 21 and 16% respectively when compared to control cells.

1.7 Effect of Norepinephrine
In another set of growth related studies norepinephrine (NRE) was used to assses the specificity of the Iso. In our studies we found that norepinephrine showed a mitogenic stimulation of 29% and 20% at 48 and 72 hrs respectively (fig. 6). The stimulation induced by the norepinephrine was less as compared to the Iso induced stimulation in the treated Dictyostelium cells (fig. 6).
Fig. 5 Growth curve of *Dictyostelium* cells treated with different concentration of aminophylline (Aph).

Fig. 6 Growth curve of norepinephrine treated *Dictyostelium* cells as compared to either control or isoproterenol treated *Dictyostelium* cells.
2 COLONY BLOTS

Stimulated cell proliferation should be accompanied by enhanced nutrient uptake. Colony morphologies were studied as one of the parameter of the cell growth. Colony morphology of *Dictyostelium* cells were studied by plating control cells and treated cells on *E. coli* seeded nutrient agar. A bacterial lawn was first established following which 1 µl of *Dictyostelium* cell (1 x 10⁷ cells) was placed on *E. coli* seeded agar plate. The appearance of a plaque reflects the feeding ability of the amoebae. As the amoebae feed on the bacteria the diameter of the plaque increases.

The Iso and AlF₄⁻ treated and control cells were placed on *E. coli* seeded nutrient agar plates, and at regular time intervals the colony imprints was taken on 0.45 µm millipore filter paper and stained with ponceau s. Plaques started to appear in both control and treated cells but the size and speed of clearing of lawn were found to be varying. Initially the size of the colony appeared similar but at 48 hrs onward the changes in time and size of colony formation became more pronounced in treated cells as compared to the control cells. The colony size of the Iso treated cells increased at 48 hrs onward and became noticeably larger at 72 hours of growth as compared to the AlF₄⁻ treated cells or the control cells. The size of the colony in Iso treated cells at 96 hrs was calculated to be 418 µm² as compared to 366.75 µm² of the control cells (fig. 7). The colony formation ability in case of AlF₄⁻ was less as compared to the control cells.

3 FOLIC ACID CHEMOTAXIS

Folic acid chemotaxis plays an important role in *Dictyostelium* as it helps it in seeking food. The natural source of food for growing amoebae, the bacteria is recognised through the folic acid chemotaxis. The *Dictyostelium* amoebae sense folic acid released by bacteria and a gradient is established by the action of folate deaminase, which the amoebae secrete into the surroundings. The gradient created by the action of folate deaminase helps in the detection and chemotaxis of the growing amoebae toward the food source. Folic acid chemotaxis was monitored in the Iso and AlF₄⁻ treated cells as compared to the control cells.
<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Control</th>
<th>200 μM (+) Iso</th>
<th>400 μM AlF₄⁻</th>
</tr>
</thead>
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<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>72</td>
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<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>96</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Fig. 7 Colony blots of control and treated *Dictyostelium* cells. Bar = 5mm
3.1 Isoproterenol
The Iso treated and the control Dictyostelium cells was plated as 1 µl (1 × 10⁷ cells) on folic acid-agar (1 × 10⁻⁵ M) and was monitored for chemotaxis. The cells were scored positive to the chemotaxis assay when the cells moved out of the droplet. The rate and number of Dictyostelium cells that were responsive to folic acid were different in treated cells as compared to the control cells. By 3-6 hr more number of Iso treated cells moved greater distance out of droplets (fig. 8b), when compared to the control cells (fig. 8a). There was considerably faster chemotaxis in case of Iso treated cells as compared to the control cells (Fig. 8b).

3.2 Aluminum fluoride
The rate of the chemotaxis of AlF₄⁻ treated Dictyostelium cells was slower as compared to the control cells. At 3-6 hr there were fewer cells that moved out of the droplet in the AlF₄⁻ treated cells as compared to the control cells. At 6 hr though there was more cells that moved out of the droplet but still considerably less as compared to the control cells (fig. 8c).

4 FOLATE DEAMINASE (FDA) ACTIVITY
During the growth of the Dictyostelium folate deaminase is one of the important enzyme that is secreted extracellularly which is necessary for the detection of folic acid secreted by bacteria as its food source in wild. Folate deaminase is involved in the breakdown of folic acid to 2-deaminofolic acid. The folate deaminase was assayed for its activity in control and treated Dictyostelium cells for 12 hrs at 2 hrs intervals.

4.1 Isoproterenol
Folate deaminase activity was assayed in control and treated cells. Dictyostelium cells treated with Iso for 30 min showed higher FDA activity and its peak activity was found to be 4.5 FDA units at 4 hr and the FDA activity subsequently decreased with time. Iso treated Dictyostelium cells showed 3.5 folds stimulation of FDA activity as compared to control cells at 4 hr (fig. 9).
Fig. 8 Folic acid chemotaxis in control and treated Dictyostelium cells. hrs indicates time from initiation of starvation.

A) Control, B) 200 μM (+) Iso, C) 400 μM AlF$_4^-$.

Bar = 100 μM
Fig. 9 Folate deaminase (FDA) activity in control and treated *Dictyostelium* cells.
4.2 Aluminum Fluoride

*Dictyostelium* cells treated with AlF$_4^-$ showed lesser FDA activity as compared to control cells. The AlF$_4^-$ treated cells showed 50% inhibition of folate deaminase activity at 2, 4, and 6 hrs interval as compared to the control cells (fig. 9).

5 ENDOCYTOSIS

Endocytosis plays an important role in the nutrition of the *Dictyostelium* cells. They obtain their nutrition either by fluid phase pinocytosis or by the phagocytosis of solid food, for example, bacteria. The pinocytosis and the phagocytosis were measured in the treated *Dictyostelium* cells.

5.1 PINOCYTOSIS

Pinocytotic uptake of liquid nutrient was measured at regular interval in control and treated cells by administering FITC-dextran as a fluid phase marker.

5.1 (a) Isoproterenol

The *Dictyostelium* cells treated with Iso for 30 min showed enhanced pinocytotic uptake of FITC-dextran as compared to the control cells. Immediately after treatment the Iso treatment showed 6% enhanced pinocytotic uptake of FITC-dextran. After 24 hrs growth of Iso treated cells showed 16% increased pinocytosis as compared to the control cells. At 48 hr and 72 hrs the Iso treated cells showed pinocytotic levels similar to that of control cells (fig. 10).

5.1 (b) Aluminum fluoride

The *Dictyostelium* cells when treated with 400 µM AlF$_4^-$ showed decreased activity in the pinocytotic uptake of FITC-dextran as compared to the control cells. The pinocytosis of FITC-dextran in AlF$_4^-$ treated *Dictyostelium* cells when assayed immediately after treatment and at 24 hrs was found to be 16% less when compared to control cells (fig. 10). At 48 hrs the pinocytosis in treated cells was found to be 14% higher as compared to the control cells.
5.2 PHAGOCYTOSIS
Phagocytosis was measured at regular interval upto 72 hrs in control and the treated cells by administering FITC-labelled bacteria. The *Dictyostelium* cells take up bacteria by phagocytosis. The uptake of FITC-labelled bacteria was taken as phagocytotic index.

5.2 (a) Isoproterenol
*Dictyostelium* cells from mid log phase were treated with 200 μM (+) Iso for 30 min and phagocytosis were measured. Iso treated *Dictyostelium* cells showed enhanced phagocytotic uptake of FITC-labelled *E. coli* in time dependent manner. At 24 and 48 hrs it showed 17% and 26% enhanced phagocytotic uptake of *E. coli* respectively as compared to control cells (fig. 11).

5.2 (b) Aluminum fluoride
*Dictyostelium* cells treated with 400 μM AlF₄⁻ showed 5% stimulation in phagocytotic uptake of FITC-labeled *E. Coli* immediately after treatment but it showed 17 and 8% inhibition in phagocytotic uptake of FITC-labelled *E. coli* at 24 and 48 hrs respectively as compared to control cells (fig. 11).

6 MACROMOLECULAR SYNTHESIS
DNA and proteins constitute bulk of the living organisms, the response of the organism to any physical and chemical agents is reflected in the pattern of the macromolecular synthesis.

6.1 DNA SYNTHESIS
The result of enhanced cell proliferation in treated cells were correlated with the ³H-thymidine incorporation studies. The DNA synthesis was studied by allowing the control and treated *Dictyostelium* cells to grow in axenic media containing ³H-thymidine for one hr following which the radioactivity was measured in TCA insoluble fraction.

6.1.1 Uptake
Following the treatment of *Dictyostelium* cells with Iso or AlF₄⁻, tritium labeled thymidine uptake was monitored in *Dictyostelium* cells.
Fig. 10 Pinocytotic uptake of FITC-dextran in control and treated Dictyostelium cells.

Fig. 11 Phagocytotic uptake in control and treated Dictyostelium cells.
6.1.1 (a) Isoproterenol
In Iso treated cells there was 17% and 25 % reduced uptake of $^3$H-thymidine at 0 and 24 hrs respectively as compared to the control cells (fig. 12).

6.1.1 (b) Aluminum fluoride
The cells treated with AlF$_4^-$ showed increased uptake of labelled $^3$H-thymidine as compared to the control cells (fig. 12).

6.1.2 Incorporation
The incorporation of $^3$H-thymidine was taken as an index of DNA synthesis activity in growing Dictyostelium cells.

6.1.2 (a) Isoproterenol
Iso treatment led to enhanced $^3$H-thymidine incorporation as compared to the control cells. Iso treatment showed 50% and 10% enhanced $^3$H-thymidine incorporation at 24 and 48 hrs as compared to the control cells (fig. 13).

6.1.2 (b) Aluminum fluoride
The cells treated with AlF$_4^-$ showed 25-30% increase incorporation of $^3$H-thymidine at 0 and 24 hrs when compared to the control cells (fig. 13).

6.2 PROTEIN SYNTHESIS
The protein synthesis was studied by allowing the control and treated cells to grow in axenic media containing $^3$H-leucine for one hr following which the radioactivity was measured in TCA insoluble fraction.

6.2.1 Uptake
The $^3$H-leucine uptake was monitored in the cells treated with Iso or AlF$_4^-$ for 30 min.

6.2.1 (a) Isoproterenol
The Iso treated cells showed approximately 30 and 40% decreased uptake of the $^3$H-leucine at 0 and 24 hrs as compared to the control cells. There was recovery of uptake of $^3$H-leucine to the level of control cells at 48 hrs (fig. 14).
6.2.1 (b) Aluminum fluoride
The cells treated with AlF$_4^-$ led to 20-15 % decreased uptake of $^3$H-leucine at 0 and 24 hrs as compared to control cells (fig. 14).

6.2.2 Incorporation
The Iso treated cells showed enhanced incorporation of $^3$H-leucine in protein by approximately 60% and 10 % as compared to the control cells (fig. 15).

6.2.2 (a) Isoproterenol
When the Iso treated Dictyostelium cells were post treated with Pro it still showed increased $^3$H-leucine incorporation when compared to the control cells (fig. 15).

6.2.2 (b) Aluminum fluoride
The cells treated with AlF$_4^-$ led to 10 % increased incorporation of $^3$H-leucine at 0 and 24 hrs as compared control cells (fig. 15).

7 ANALYSIS OF THE CYTOSKELETAL PROTEINS
Cytoskeleton proteins are the fibrous proteins, which forms the architecture of the cell. It plays important role in the number of physiological aspects for example it in involved in the cellular movement, defining polarity of cell, phagocytosis, in the cell division. The cytoskeleton proteins were isolated from control and the treated cells as Triton-X 100 insoluble fraction and were electrophoresed in 7 % polyacrylamide gel.

The cytoskeletal proteins were isolated from cells treated with Iso, AlF$_4^-$ and control cells and were analysed on SDS-PAGE. It was found that there was decreased association of actin with cytoskeletal fraction in Iso treated cells (fig. 16, lane 3). The AlF$_4^-$ showed increased density of band indicating increased association of actin with the cytoskeletal fraction (fig. 16, lane 2). The gels were scanned and densitometric analyses were carried out using Fuji Film Image reader. From densitometric studies we found that the Iso and AlF$_4^-$ treated cells showed 60 % and 76 % actin content respectively as compared to the control cells.
Fig. 12  Uptake of $^3$H-thymidine in control and treated *Dictyostelium* cells.

Fig. 13  Incorporation of $^3$H-thymidine in control and treated *Dictyostelium* cells.
Fig. 14  Uptake of $^3$H-leucine in control and treated Dictyostelium cells.

Fig. 15  Incorporation of $^3$H-leucine in control and treated Dictyostelium cells.
Fig. 16  SDS-PAGE analysis of cytoskeletal proteins isolated from control and treated Dictyostelium cells as tritonX-100 insoluble fraction.
M – Molecular weight marker, Lane 1– Control, Lane 2 – AlF$_4^-$, Lane 3 – Iso
Table III: A comparative account of effects of isoproterenol and aluminum fluoride on different growth related activities of *Dictyostelium discoideum*.

<table>
<thead>
<tr>
<th>Growth Parameter</th>
<th>Isoproterenol 200 µM (+)</th>
<th>Aluminum fluoride 400 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitogenic stimulation (48 hrs)</td>
<td>142 %</td>
<td>122 %</td>
</tr>
<tr>
<td>Colony Blots (Area) (72 hrs)</td>
<td>417.40 µm²</td>
<td>304.67 µm²</td>
</tr>
<tr>
<td>Folic acid Chemotaxis</td>
<td>++++&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Folate Deaminase Activity (4 hr)</td>
<td>266 %</td>
<td>52 %</td>
</tr>
<tr>
<td>Endocytosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Pinocytosis (24 hr)</td>
<td>116 %</td>
<td>16 %</td>
</tr>
<tr>
<td>b) Phagocytosis (24 hr)</td>
<td>118 %</td>
<td>17 %</td>
</tr>
<tr>
<td>Protein Synthesis</td>
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</tr>
<tr>
<td>a) Uptake (24 hr)</td>
<td>70 %</td>
<td>93 %</td>
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<tr>
<td>b) Incorporation (24 hr)</td>
<td>120 %</td>
<td>110 %</td>
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<tr>
<td>DNA Synthesis</td>
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<td></td>
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<tr>
<td>a) Uptake (24 hr)</td>
<td>75 %</td>
<td>116 %</td>
</tr>
<tr>
<td>b) Incorporation (24 hr)</td>
<td>152 %</td>
<td>131 %</td>
</tr>
<tr>
<td>Actin</td>
<td>60 %</td>
<td>76%</td>
</tr>
</tbody>
</table>

% - Percentage of control, ++++<sup>a</sup> - strongly positive, +<sup>b</sup> - slightly higher than control.
Conclusions

In our growth-related studies viz., growth, colony morphology, chemotaxis, macromolecular syntheses, and endocytotic activities were monitored in Iso and AlF$_4^-$ treated Dictyostelium cells, and most of the physiological processes was found to be enhanced.

All the above discussed growth parameters demonstrate that Iso and AlF$_4^-$ are stimulator of the vegetative growth of Dictyostelium amoebae. The mitogenic stimulation of the Iso treated cells showed that it is dose and isomer dependent. 200 µM (+) Iso showed maximum stimulation of 42 % as compared to growth at 48 hrs of growth as compared to 22% stimulation showed by AlF$_4^-$ treated cells.

All growth related parameters were found to be stimulated in case of Iso treated cells as compared to the control cells. Use of antagonist (propranolol) to Iso was found to reverse the mitogenic stimulation caused by Iso treatment. Epinephrine was found to show similar growth stimulation like that of Iso. Adenylate cyclase could be the potential target for the both Iso and AlF$_4^-$ that leads to mitogenic stimulation.
II DEVELOPMENTAL STUDIES

Depletion of food source leads to the starvation of the Dictyostelium cells to the developmental phase from the growth phase. In laboratory conditions starvation was induced by washing the cells of nutrient media with P buffer. There were two sets of experiment done, viz., on growing Dictyostelium cells and on the starving Dictyostelium cells. The cells were then plated on P-agar to study its different developmental changes. Various parameter under which the developmental aspects of treated cells of Dictyostelium discoideum studied were,

(i) cAMP chemotaxis
(ii) cAMP dependent extracellular phosphodiesterase
(iii) Cell Streaming
(iv) EDTA stable cell contact
(v) Aggregates
(vi) Slug stage
(vii) Mexican hat stage
(viii) Fruiting body formation

8 CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE (CYCLIC-AMP) CHEMOTAXIS

cAMP chemotaxis is an important cellular phenomenon during the developmental phase of the Dictyostelium cells. This is triggered as consequence of the starvation and helps in aggregation of spatially scattered Dictyostelium cells leading to the formation of multicellular aggregates. Control and treated Dictyostelium cells were plated immediately after treatment as small droplets on cAMP agar (1 μM) at a cell density of 3 x 10^7 cells/ml and were monitored for chemotaxis. The cells were scored positive when they moved out of droplet.

8.1 Isoproterenol

Dictyostelium cells treated with 200 μM (+) Iso for 30 min showed enhanced cAMP chemotaxis. Initially at 6 hrs the rate of chemotaxis appeared similar to
the control cells but at 12 hrs larger number of cells moved out from the droplets which was higher as compared to the control cells. The distance covered by the treated cells were also more when compared to the control cells (fig. 17b).

8.2 Aluminum fluoride

*Dictyostelium* cells when treated for 30 min with 400 μM AIF$_4^-$ led to decline in the cAMP chemotaxis as compared to the control cells. In the AIF$_4^-$ treated cells the number of cells, which moved out of the droplets, was less as compared to the control cells (fig. 17c).

9 CYCLIC 3',5'AMP DEPENDENT EXTRACELLULAR PHOSPHODIESTERASE (ePDE) ACTIVITY

Extracellular phosphodiesterase activity was assayed in control and treated *Dictyostelium* cells at 2 hr intervals 4, 6, 8, and 10 hrs after the initiation of starvation.

9.1 Isoproterenol

The Iso treated *Dictyostelium* cells were monitored for their extracellular phosphodiesterase activity. The treated cells showed twice the ePDE activity as compared to that of control cells at 8 hrs of development (fig 18).

9.2 Aluminum fluoride

*Dictyostelium* cells treated with AlF$_4^-$ for 30 min showed lesser ePDE activity as compared to control cells. The AlF$_4^-$ treated cells showed 20% reduced ePDE activity as compared to the control cells (fig. 18) at 8 hrs of development. However AlF$_4^-$ treated *Dictyostelium* cells showed marginal increase in ePDE activity at 8 hr as compared to control cells.

10 CELL STREAMING

There were two sets of experiment done, (i) on growing and (ii) developing *Dictyostelium* cells were treated with Iso and AlF$_4^-$ for 30 min, the control and the treated cells were then plated on the agar plate at 22°C and their morphogenesis were studied. Control cells completed their morphogenesis (fruiting body formation) in 30 hours of plating on the agar plate.
Fig. 18 Extracellular phosphodiesterase (ePDE) activity (nM cAMP/ml/min) in control and treated Dictyostelium cells.
Control and treated cells showed streaming within 7-10 hrs of development. In control the aggregates appears at 8-10 hrs of development (fig. 19a).

10.1 Isoproterenol
The Iso treatment induced faster streaming, it showed well formed, and large pseudopodia formation similar to the control cells. The Iso treated growing cells showed streaming after 6-7 hrs of development (fig. 19b). The developing cells showed streaming after 7-9 hrs of starvation (fig. 20b).

10.2 Aluminum fluoride
Treatment of the Dictyostelium cells with AlF<sub>4</sub><sup>−</sup> led to formation of smaller streaming and showed delayed streaming at 14-16 hrs (fig. 19c) whereas the developing cells when treated with AlF<sub>4</sub><sup>−</sup> showed early streaming and appeared at 12-13(fig. 20c).

11 EDTA STABLE CELL CONTACT
The control and the treated Dictyostelium cells were suspended in phosphate buffer and the formation of EDTA stable cell contact was monitored at regular time intervals.

11.1 Isoproterenol
Dictyostelium cells exposed to 200 μM (+) Iso for 30 min and the formation of EDTA stable cell contact were monitored after every2 hrs. The contact formation was faster in the Iso treated cells, it showed EDTA stable cell contact at 4 hrs formation and at 8 hrs one could see large contact formation 70 % of the cells showed the EDTA stable contact formation (fig. 21b). The control cells also showed large cell-to-cell EDTA stable contact formation but were smaller, 60 % of the cells showed EDTA stable contact formation at 8 hrs (fig. 21a).

11.2 Aluminum fluoride
The treatment of Dictyostelium cells with AlF<sub>4</sub><sup>−</sup> for 30 min showed slower and less number of EDTA stable contact formation as compared to the
Fig. 19 Cell streaming in growing *Dictyostelium* cells.

A) Control; B) 200 μM (+) Iso; C) 400 μM AlF$_4^-$ Bar = 100 μM
Fig. 20  Cell streaming of developing Dictyostelium cells.
A) Control; B) 200 μM (+) Iso; C) 400 μM AlF$_4^-$ Bar = 100 μM
Fig. 21 EDTA stable cellular aggregates in Dictyostelium cells.

A) Control; B) 200 μM (+) Iso; C) 400 μM AlF$_4^-$ . Bar = 100 μM
control cells. Only 30% of the treated Dictyostelium cells exhibited less EDTA stable contact formation (fig. 21c).

12 AGGREGATES
Streaming of the cell lead to the formation of the aggregates. The aggregates appear at 12-14 hrs of development in the control cells. The aggregates are large globular structure measuring about 1113 μM.

12.1 Isoproterenol
Growing cells treated with Iso led to the formation of larger aggregate measuring about 1290 μm formation in the treated cells. The aggregates appear at 9-11 hrs of the development (fig. 22b). The developing cells when treated with Iso led to formation of large aggregates of 1210 μm size and appeared earlier as compared to that of treated growing cells, at 6-8 hrs of development (fig. 23b).

12.2 Aluminum fluoride
Formation of aggregates in the AlF₄⁻ treated cells showed smaller size (859 μm) and they appeared in larger number. There was a delay between 3-4 hr before the appearance of aggregates as compared to Iso treated cells (fig. 22c). The developing cells led to formation of smaller aggregates (573 μm) and they appeared in large number at 11-13 hrs of starvation (fig. 23C).

13 SLUGS
The aggregates move as a unit in search of favourable light and humidity condition for their further development, and this represents the slug stage. In control cells the slug appears at 14-16 hrs of development (fig. 24a) and were large sized (580 μm).

13.1 Isoproterenol
The slugs appear earlier than control in the Iso treated cells, between 13-15 hr of development. The slugs are well formed (501 μm) and larger in the size and appeared bloated (fig. 24b). In case of treated cells treated led to
Fig. 22 Formation of aggregates of treated growing Dictyostelium cells.  
A) Control; B) 200 μM (+) Iso; C) 400 μM AlF₄⁻.  Bar = 100 μM
Fig. 23  Formation of aggregates in developing *Dictyostelium* cells.  
**A)** Control; **B)** 200 μM (+) Iso;  **C)** 400 μM AlF$_4^-$  
Bar = 100 μM
formation of slightly smaller sized slugs as compared to the control cells (fig. 25b) and the slugs appeared between 11-13 hrs of development.

13.2 Aluminum fluoride
The treatment of *Dictyostelium* cells with AlF$_4^-$ leads to delay in the appearance of the slugs. They appear at 15-17 hrs of the development. The number of the slugs was more as compared to either control or the Iso treated cells and was of smaller size (128 µm) (fig 24c). In case of developing cells treated with AlF$_4^-$ led to formation of smaller slugs but were relative larger sized as compared to the AlF$_4^-$ treated growing cells. The slugs appeared at 16-18 hrs of development (fig. 25c).

14 MEXICAN HAT
After brief migration the slugs in search of favorable conditions settle down at a suitable place and form a globular structure giving an appearance of mexican hat. The mexican hat is formed immediately after the slug settles down for further development. The slugs appear at 17-20 hrs of the development (fig. 26a)

14.1 Isoproterenol
Iso treatment led to faster appearance of the mexican hat stage and, they are seen at 16-18 hrs of the development (fig 26b). The mexican hat were large sized and appeared at 16-18 hrs of development. The Iso treated developing cells led to earlier appearance of large mexican hat, it appeared at 15-16 hrs of development (fig. 27b).

14.2 Aluminum fluoride
AlF$_4^-$ treatment causes a delayed appearance of the mexican hat stage as they appeared at 23-25 hrs of development. They are of smaller size and appear as oval shaped (fig. 26c). The developing cells when treated with AlF$_4^-$ led to formation of relatively large mexican hat and it appeared at 19-21 hrs of development (fig. 27c).
Fig. 24 Slugs in treated growing *Dictyostelium* cells.
A) Control; B) 200 μM (+) Iso; C) 400 μM AlF₄⁻. Bar = 100 μM
Fig. 25 Slugs in developing *Dictyostelium* cells.

A) Control; B) 200 μM (+) Iso; C) 400 μM AlF$_4^-$.

Bar = 100 μM
Fig. 26  Mexican hat treated growing *Dictyostelium* cells.

A) Control; B) 200 μM (+) Iso;  C) 400 μM AlF$_4^-$  Bar = 100 μM
Fig. 27  Mexican hat of treated developing *Dictyostelium* cells.
A) Control; B) 200 µM (+) Iso; C) 400 µM AlF$_4^-$  Bar = 100 µM
FRUITING BODIES

After a brief spell of mexican hat stage the Dictyostelium cells proceeds to the formation of the fruiting bodies. The mexican hat, in turn, forms a fruiting body containing a mass of spore (sorus) cells supported by a column of stalk cells. The fruiting bodies appear after 25-28 hrs of development (fig. 28a).

15.1 Isoproterenol

Fruiting bodies appear at 24-26 hrs of development in the Iso treated cells. The fruiting bodies appear to be similar to the control cells but some sorii are larger as compared to the controls (fig 28b). In case of developing cells the fruiting bodies appeared earlier as compared to the control cells the sorii was bigger as compared to the control cells, but the stalk were stout and smaller sized (fig. 29b). The fruiting bodies began to appear at 20-21 hrs of development.

15.2 Aluminum fluoride

Fruiting bodies in the AlF 4− treatment appear at 27-30 hrs after development. The development is slower in case of the AlF 4− treated cells. The fruiting bodies are well formed but have a longer stalk (fig. 28c). In case of developing cells treated with AlF 4−, led to formation of normal sized fruiting bodies with a slender stalk. The formation of fruiting bodies was slower and began to appear at 23-24 hrs of development (fig. 29c).

Conclusions

In our development related studies viz., cAMP chemotaxis, cAMP dependent phosphodiesterase activity, EDTA stable cell contact, cell streaming, aggregates, slug and fruiting bodies formation were monitored in Iso and AlF 4− treated Dictyostelium cells. All the above developmental activities are enhanced Iso treated Dictyostelium discoideum cells. The Iso treated cells showed higher cAMP and ePDE activity and the developmental process are completed in 24 hrs (table IV). The developmental activities in AlF 4− treated cells are delayed considerably as compared to the control cells (table IV).
Fig. 28  Fruiting bodies in treated growing *Dictyostelium* cells.

**A)** Control; **B)** 200 μM (+) Iso;  **C)** 400 μM AlF$_4$$.  Bar = 100 μM
Fig. 29  Fruiting bodies in treated developing *Dictyostelium* cells.
A) Control; B) 200 μM (+) Iso; C) 400 μM AlF$_4^-$  Bar = 100 μM
Table IV – Timing and morphological features of developmental stages of the control and treated *Dictyostelium discoideum* cells.

h - Approximate time of appearance

<table>
<thead>
<tr>
<th>Developmental stages</th>
<th>Control</th>
<th>Isoproterenol (+) 200 µM</th>
<th>Aluminum fluoride 400 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streaming</td>
<td>6-8 h</td>
<td>4-5 h fast, stout and well branched</td>
<td>7-9 h delayed, smaller and diffused branches</td>
</tr>
<tr>
<td>Aggregates</td>
<td>10-12 h</td>
<td>6-8 h big and well formed</td>
<td>12-13 h smaller and loosely formed</td>
</tr>
<tr>
<td>Slugs</td>
<td>15-16 h</td>
<td>12-13 h large, bloated shape</td>
<td>16-18 h small and large number</td>
</tr>
<tr>
<td>Mexican Hat</td>
<td>18-20 h</td>
<td>15-16 h big</td>
<td>19-20 h small</td>
</tr>
<tr>
<td>Fruiting Body</td>
<td>22-24 h</td>
<td>20-21 h big sorus, stout stalk</td>
<td>23-24 h small, slender stalk</td>
</tr>
</tbody>
</table>
III CYTOMORPHOLOGICAL STUDIES
The following cytomorphological studies of the control and treated Dictyostelium cells were investigated.

(i) Live cell observations
(ii) Light microscope observations
(iii) Electron microscopic studies

16 LIVE CELL OBSERVATIONS
Control and treated Dictyostelium cells suspensions were placed in a multiwell test plate for live cell observations.

16.1 Isoproterenol
Following Iso treatment for 30 min light microscopic observations showed that the treated cells assumed spherical shapes and remained loosely bound to the substratum immediately after treatment. At 24 hrs few large cells appears and at 48 hrs onward many large and rounded cells were observed among the treated cell population (fig. 30). At 48 hrs the Iso treated cells showed larger cells, which are approximately double the sizes of the normal cells. Many rounded cells could be noticed among the amoeboid shaped cells. The size of larger cells in Iso treated cells was 1573 ± 492 \( \mu \text{m}^2 \) at 48 h as compared to 305 ± 84 \( \mu \text{m}^2 \) control cells, i.e. triple the size of the control cells.

16.2 Aluminum fluoride
There is significant increase in cell number (40 – 45% of control) in case of Iso treated cells when compared with control cells. AlF\(_4\)\(^-\) treated cells also showed increased cell number (10-25% of control) as compared to the control cells. The AlF\(_4\)\(^-\) treated cells also exhibited many rounded cells at 48 hrs of growth. The Iso and AlF\(_4\)\(^-\) treatment showed higher cell density as compared to the control cells at 48 hours onward after the treatment (fig. 30). In AlF\(_4\)\(^-\) treated cells the cell size was calculated to be 447 ± 58 \( \mu \text{m}^2 \), which is larger as compared to the control cells. The cell number increased dramatically in the treated cells up to 48 hrs (fig 30).
Fig. 30 Cell morphology and cell density of control and treated *Dictyostelium* cells at different time intervals. Arrow indicates the large cells. Note large no of large cells in Iso treated *Dictyostelium* cells. Bar= 20 μM
17 LIGHT MICROSCOPIC OBSERVATIONS OF THIN CELL SECTIONS
Thin sections (1-2 μM) of araldite embedded control and treated cells were prepared and were stained with toluidine blue and were observed under light microscope at high magnification.
The control cells appeared to show large number of intact vacuoles (fig. 31a). The Iso treated cells showed fewer vacuoles, which are large (fig. 31b). The AIF₄⁻ treated cells appeared to show fewer vacuoles (fig. 31c).

18 SCANNING ELECTRON MICROSCOPIC (SEM) OBSERVATIONS
Treated Dictyostelium cells were resuspended in P-buffer and were fixed in glutaraldehyde, air-dried and were then gold sputter coated. These cells were observed under scanning electron microscope.
Scanning electron micrographs of control vegetative cells showed amoeboid cells with fine filopodial projections from the cellular membrane. On closer observations the cellular membrane showed large ruffling (fig 32a).

18.1 Isoproterenol
Scanning electron micrograph of Iso treated cells showed large rounded cells. There was lot of surface projections and the membrane appeared ruffled. Few large indations in the membrane could be seen, may be the pinosomal formation structure. Large number of dumbel shaped cells could be seen, those are the cells that are undergoing cell division. Lot of smaller clumps of 2 or 3 cells could be seen (fig 32b).

18.2 Aluminum fluoride
In case of AIF₄⁻ treated cells showed spherical shaped smaller cells. The membranes showed smaller ruffleled structure in contrast to the large ruffled appearance of the Iso or the control vegetative cells (fig 32c).

19 TRANSMISSION ELECTRON MICROSCOPIC OBSERVATIONS
The ultra thin sections of vegetative Dictyostelium cells observed under TEM showed characteristic irregular amoeboid shape, displaying some small filopodia like structure projecting from the membrane. The cytoplasm showed
Fig. 31  Photomicrographs of toludine blue stained thin section (1μ) of control and treated *Dictyostelium* cells at 48 hrs of growth.

A) Control; B) 200 μM (+) Iso; C) 400 μM AlF<sub>4</sub><sup>−</sup>. Arrow indicates the large vacuoles.  Bar = 10 μM
Fig. 31 (Legend on the facing page)
Fig. 32 Scanning electron micrographs of control and treated Dictyostelium cells.
A) Control; B) 200 μM (+) Iso; C) 400 μM AlF$_4^-$ 4000X
large number of electron dense granules and a prominent large nucleus. Various cellular organelles e.g., mitochondria, endoplasmic reticulum (fig 33a).

19.1 Isoproterenol
TEM of Iso treated cells showed large number of large vacuoles concentrated towards the periphery of the cells. The cellular morphology was irregular. In the cytoplasm one could see few large vacuoles situated near the periphery of the cell. Nucleus in the Iso treated cells is well developed. In some cells, binucleated conditions were seen. There is an increased presence of golgi bodies, which were uniformly distributed in the cytosol. The endoplasmic reticulum is well developed. Lots of electron dense granules could be found scattered in cytosol (fig 33b).

19.2 Aluminum fluoride
TEM of AlF$_4^-$ treated cells showed an irregular cellular morphology. There were few smaller vacuoles scattered in the cytoplasm (fig 33c). The nucleus was large sized and number of vacuoles were seen in cytosol. Few golgi bodies could be seen in cytosol.
Fig. 33  Transmission electron micrographs of control and treated growing *Dictyostelium* cells.  

A) Control; B) 200 μM (+) Iso; C) 400 μM AlF$_4^-$.

Arrow indicates the binucleated structure, G-golgi bodies, **1500X**