MATERIALS AND METHODS
I MATERIAL

Dictyostelium discoideum which is popularly known as cellular slime mould, is the experimental organism for the present study. The axenic strain (Ax2) obtained from Dr. Robert Kay, Medical Research Council, Cambridge, U.K., was used for all experiments. The culture methods for the maintainance of Ax2 strain are described below.

II METHODS

1 PREPARATION OF CULTURE MEDIA AND BUFFERS

1.1 Preparation of Axenic Medium

The axenic medium has the following ingredients (Ashworth and Watts, 1970):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone (Difco)</td>
<td>14.3 gms</td>
</tr>
<tr>
<td>Yeast extract (Hi-Media)</td>
<td>7.5 gms</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.2H$_2$O (S.d.Fine-chem)</td>
<td>0.616 gms</td>
</tr>
<tr>
<td>KH$_2$PO$_4$.2H$_2$O (S.d.fine-chem)</td>
<td>0.486 gms</td>
</tr>
<tr>
<td>Maltose (E.Merk)</td>
<td>18.0 gms</td>
</tr>
</tbody>
</table>

The above ingredients were added to double distilled water to make the volume 1 litre and pH was adjusted to 6.7. 50 ml of axenic medium was dispensed into 250 ml capacity Ehrlenmeyer flasks and autoclaved at 15 lbs pressure for 20 min.
1.2 Preparation of Luria Broth

Luria Broth has the following ingredients:

- NaCl (Glaxo) : 10 gms
- Bactopeptone (Difco) : 10 gms
- Yeast extract (Hi-Media) : 5 gms

The above ingredients were added to double distilled water to make the volume 1 litre and the pH was adjusted to 7.5. The broth was autoclaved at 15 lbs for 20 min.

1.3 Preparation of Nutrient Agar (N-Agar)

The Nutrient Agar has the following ingredients:

- Glucose (Glaxo) : 1 gm
- Proteosepeptone (Difco) : 1 gm
- Agar (Hi-Media) : 20 gms

The above ingredients were added to 1 litre of Sorenson's phosphate buffer (pH 6.3) and autoclaved. 20 ml of the medium was poured into 90 mm sterile petri plates under sterile conditions and allowed to polymerize (Sussman, 1987).

1.4 Preparation of Non-nutrient Agar

Non-Nutrient Agar or Phosphate Agar (P-Agar) was prepared in Sorenson's phosphate buffer (pH 6.3). 20 gms of agar (Hi-Media) was added to 1 litre of phosphate buffer (pH 6.3) and autoclaved. 20 ml of the medium was poured
into 90 mm sterile petri plates and allowed to polymerize.

1.5 Preparation of Sorenson's phosphate buffer (P-buffer)

0.017 M Sorenson's phosphate buffer (P-buffer) was made by dissolving the following salts in 1 litre of double distilled water and pH was adjusted to 6.3.

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} : & \quad 2.37 \text{ gms} \\
\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} : & \quad 7.25 \text{ gms}
\end{align*}
\]

2. CULTURING OF DICTYOSTELIUM DISCOIDEUM CELLS

Ax2 strain of Dictyostelium discoideum can be grown either in a liquid suspension i.e., in axenic medium or on a solid substratum i.e., on a nutrient agar plate seeded with bacteria as food source,

2.1 Growth of Ax2 cells in axenic medium

Spores of Ax2 cells were inoculated into 10 ml of axenic medium in a 100 ml capacity Ehrlenmeyer flask and incubated at 22°C in an orbital shaker at 120 rpm. After 4-5 days of incubation, the germinated amoebae were inoculated into 10 ml of fresh axenic medium. By 2-3 days the amoebae will grow to mid-log phase and serve as the preculture. This preculture was used to inoculate 50 ml fresh axenic medium in a 250 ml capacity Ehrlenmeyer flask to give an initial cell density of $4 \times 10^5$ cells/ml. Fresh inoculations were made every day to provide a regular supply of Ax2 cells.

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2.2 Growth and development of Ax2 cells on Nutrient Agar

_E. coli_ was inoculated into sterile Luria Broth and was grown at 37°C for over night. 0.2 ml of _E. coli_ was spread evenly with a glass spreader on a Nutrient Agar plate. 0.1 ml of Ax2 cells were uniformly spread on the bacterial lawn under sterile conditions. These plates were incubated at 22°C (Sussman, 1987).

2.3 Development of Ax2 cells on Non-Nutrient Agar

Exponentially growing _Dictyostelium discoideum_ cells were harvested from axenic medium by centrifuging at 2000 rpm for 2 min. These cells were washed two times in ice cold P-buffer and resuspended in P-buffer at a final density of 5x10^7 cells/ml. 1 ml of this cell suspension was evenly spread on P-Agar plates and were maintained at 22°C.

2.4 Spore collection and inoculation

Spores were collected from P-Agar plates containing fully developed fruiting bodies. Agar plates containing fruiting bodies were inverted over another plate containing sterile P-buffer and gently tapped. The fallen spores were collected in P-buffer and stored in eppendorf tubes at -80°C.

For inoculation the eppendorf tubes containing spores were thawed. The spores were thoroughly washed in P-buffer
and resuspended in 10 ml of axenic medium at a density of 2x10^5 spores/ml in 100 ml capacity Ehrlenmeyer flask. Filter sterilised Streptomycin sulphate (E.Merk) was added to give a final concentration of 500 µg/ml. The flask containing spores was given heat shock at 45°C for 25 min and was placed on an orbital shaker at 120 rpm (22°C), (Sussman, 1987).

In the present study the action of cisplatin on Dictyostelium discoideum was studied by selecting the following parameters.

(I) Growth.

(II) Morphogenesis.

(III) Cytomorphology.

3. CISPLATIN TREATMENT OF DICTYOSTELIUM DISCOIDEUM

3.1 Short term treatment

Growing Dictyostelium discoideum cells were harvested by centrifuging at 2000 rpm for 2 min and were given a wash in P-buffer. These cells were resuspended in P-buffer. Appropriate amount of cisplatin stock solution was added to give a final concentration of 100 and 200 µg/ml cisplatin and a final cell density of 5x10^6 cells/ml. After 1 hr treatment with cisplatin the cells were washed three times with ice-cold P-buffer. Growth and development of cisplatin treated cells were monitored separately either by resuspending the cells in fresh axenic medium or by
spreading on P-Agar.

3.2 Long term treatment

Appropriate amount of filter sterilised cisplatin stock (1000 ug/ml) solution was added directly to the axenic medium containing Dictyostelium discoideum cells at an initial density of 5x10⁶ cells/ml, to give a final concentration of 50 and 100 ug/ml of cisplatin. Growth and development of Dictyostelium cells were monitored separately after 24 hr exposure to cisplatin.
A. STUDIES ON GROWTH

1. SURVIVAL OF DICTYOSTELIUM DISCOIDEUM CELLS TREATED WITH CISPLATIN AT DIFFERENT PHASES OF GROWTH CYCLE

The lag phase (2x10⁶ cells/ml), mid log (5x10⁶ cells/ml) and stationary phase (1x10⁷ cells/ml) cells were treated with cisplatin. Survival of cells was monitored by staining with 0.1% trypan blue for dye exclusion test and cells were counted using a haemocytometer.

1.1 Short term treatment

Growing Dictyostelium cells at different growth phases were treated with 100 and 200 μg/ml cisplatin for 1 hr. These Cisplatin treated cells were resuspended in fresh axenic medium at an initial density of 5x10⁶ cells/ml and their survival monitored at 24 hr intervals till 96 hrs.

1.2 Continuous treatment

- Dictyostelium cells of different growth phases were grown in the continuous presence of 50 and 100 μg/ml cisplatin. Survival was monitored at 24 hrs intervals till 96 hrs.

2. CISPLATIN TREATMENT OF SPORES

2.1 Short term treatment

Dictyostelium spores were treated with 100 and 200 μg/ml of cisplatin for 1 hr and were washed thoroughly.
They were resuspended in axenic medium at an initial density of 5x10^6 spores/ml. Germination was monitored by counting ungerminated spores in a haemocytometer at 24 hr intervals till 72 hrs.

2.2 Continuous treatment

Appropriate amount of filter sterilised cisplatin (1000 μg/ml stock) solution was directly added to axenic medium containing Ax2 spores (5x10^6 spores/ml) to give a final cisplatin concentration of 50, 100 and 200 μg/ml. Germination was monitored by counting ungerminated spores using a haemocytometer at 24 hr intervals till 72 hrs.

3. COLONY MORPHOLOGY OF CISPLATIN TREATED CELLS

The colony morphology of Dictyostelium cells was studied by plating the cells on nutrient agar and preparing colony blots. The control and cisplatin treated cells were placed as small droplets on a nutrient agar plate, which was evenly spread with E.coli and incubated at 22°C. When plaques started to appear on E.coli lawn, Millipore filters (Millipore(I), 0.45 μm) were placed on top of a plaque for 3 min and then immediately frozen in liquid nitrogen. The filters were stained with 0.2% Ponceau'S'Red (Sigma) for 5 minutes. These filters were washed thoroughly in distilled water. The filters were then fixed in 3% TCA solution for 3 min and then air dried (Bozzaro et al., 1987).
4. FOLIC ACID CHEMOTAXIS

Folic acid chemotaxis was monitored in control and cisplatin treated Dictyostelium cells.

4.1 Small population assay

This assay was done according to the method of Pan, et al (1972). Control and treated cells were harvested and suspended to give a thick slurry. 2 μl of this slurry was placed as a small droplet on Folic acid-agar (1x10^-5M). The cells were scored positive to chemotaxis assay when they move out of the droplet.

4.2 Folate deaminase assay

Folate deaminase was assayed according to the method of Bernstein and Van Driel (1980). Control and cisplatin treated Dictyostelium cells were resuspended in P-buffer (pH 6.2) at a density of 1x10^7 cells/ml. Flasks containing these cells were placed on an orbital shaker (120 rpm) at 22°C under fluorescent light. 1 ml samples were removed at 2 hr intervals till 12 hrs. Cells were pelleted, supernatant was collected and assayed immediately. Enzymatic activity was assayed by following the initial rate of change of absorbance of folic acid solution at 325nm in a spectrophotometer (UV-2000, Hitachi). Samples were measured in 100 μM folic acid, 0.1M imidazole-HCl (pH 7.2) at 23°C with respect to a reference cuvette containing
similar solution without the enzyme. One unit of folate deaminase activity was defined as 1.0 nmol of folic acid converted to product (2-hydroxy 2-deamino folic acid) per minute.

5. ENDOCYTOSIS

Both pinocytosis and phagocytosis were measured in control and cisplatin treated Dictyostelium cells.

5.1 Pinocytosis

Pinocytosis was measured according the method of Vogel (1987), by administering Fluorescien isothiocynate-Dextran (FITC-dextran), FD-70S, (Sigma) as a fluid phase marker. Control and cisplatin treated cells were resuspended in fresh axenic medium at a density of \(5\times10^6\) cells/ml. FITC-dextran was added from a stock solution of 20 mg/ml to give a final concentration of 2 mg/ml and culture flasks were placed on a rotary shaker at 120 rpm (22°C). 1 ml samples were withdrawn at 30 min intervals for 3 hrs. To stop pinocytosis, each 1 ml sample was diluted 1:5 in chilled P-buffer and, cells were washed two times with chilled P-buffer and finally suspended in 3 ml of 50 mM sodium phosphste (pH 9.2). Cell number was counted and subsequently cells were lysed by addition of Triton X-100 (0.2% final concentration). The fluorescence intensity was measured by a fluorescence spectrophotometer (RF-540, Shimadzu) using exitation and emission wavelengths.
of 470 and 520 nm respectively and the pinocytosed volumes were determined from a standard curve. A standard FITC-dextran curve was made by plotting fluorescence intensities against FITC-dextran concentrations ranging from 0.1 μg/ml to 4 μg/ml.

5.2 Phagocytosis

Phagocytosis was measured by administering Fluorescein isothiocynate (FITC) labelled *E. coli* as substrate particles (Vogel, 1987).

5.2.1 Preparation of FITC labelled bacteria

FITC-bacteria were prepared by suspending exponentially grown bacteria in 50 mM sodium phosphate, pH 9.2 (O.D_{420}=20) and by adding FITC (isomer I, Sigma) stock (10 mg/ml) to give a final FITC concentration of 0.1 gm/ml. After incubation for 3 hrs at 37°C on a rotary shaker (100 rpm), bacteria were collected and washed six times by centrifuging at 7000xg for 10 min in P-buffer (pH 6.2).

5.2.2 Phagocytosis assay

Control and cisplatin treated Dictyostelium cells were resuspended in P-buffer along with FITC-bacteria (5x10⁸ bacteria/ml) in 1:200 ratio and were incubated on a rotary shaker (100 rpm) at 22°C. 1 ml samples were withdrawn at 10 min intervals for 1 hr and phagocytosis was stopped by diluting the sample with 4 ml of chilled P-buffer (pH
6.2). Uningested bacteria were removed by washing with P-buffer (pH 6.2) and pelleting Dictyostelium cells by centrifuging at 2000 rpm for 2 minutes. Washed cells were resuspended in sodium phosphate (pH 9.2) and cell numbers were determined. The cells were lysed by the addition of Triton X-100 (0.2% final concentration). The fluorescence intensity was measured by fluorescence spectrophotometer (RF-540, Shimadzu) using excitation and emission wavelengths of 470 and 520 nm respectively. The number of bacteria ingested was determined by comparison with a standard curve obtained by lysing known number of FITC-bacteria in 50 mM sodium phosphate buffer (pH 9.2) containing 1% SDS solution by heating for 2 min at 90°C and determining fluorescence intensity at excitation and emission wave lengths of 470 and 520 nm respectively (Vogel, 1987).

6. ANALYSIS OF CYTOSKELETAL PROTEINS BY SDS-PAGE

Cytoskeletal proteins were prepared as Triton X-100 insoluble fraction according to the method of Maeda (1988). Control and cisplatin treated cells were suspended at a density of 1x10^7 cells/ml in 5 ml of lysis solution containing 1% Triton X-100, 10 mM KCl, 10 mM imidazole, 2 mM sodium azide, 5 mM EGTA and 8.5 mM phosphate buffer (pH 6.2). The cell suspension was kept at room temperature (23°C) for 10 minutes with occasional agitation and the samples were then centrifuged for 4 min at 8000xg (4°C). The supernatant was discarded and the pellet was resuspended...
in 5 ml of lysis solution, incubated for 10 min at room temperature (\textdegree{}23\textdegree{}C) with occasional agitation and centrifuged for 4 min at 8000xg. The supernatant was discarded and the cytoskeletal proteins were lyophilized.

Cytoskeletal proteins prepared by above method were boiled for 1 min in 1 ml of sample buffer containing 0.125M Tris-HCl (pH-6.8), 4% sodium dodecyl sulphate, 20% glycerol and 2% mercaptoethanol. 10 \( \mu l \) of these samples were loaded on a 10% polyacrylamide gel and was electrophoresed at a constant voltage of 120 volts (Laemmli, 1970). The proteins were stained with 0.025% Coomassie brilliant blue R-250 in 40% methanol and 7% acetic acid for 2 hrs, then destained in destaining solution containing 50% methanol and 10% acetic acid.

7. MACROMOLECULAR SYNTHESIS

7.1 DNA synthesis

Control and cisplatin treated Dictyostelium cells were resuspended in axenic medium at a density of 5x10\textsuperscript{6} cells/ml. 400 \( \mu l \) of these growing cell suspensions were labelled at different time intervals (0, 6 and 12 hr after treatment) with 50 \( \mu Ci/ml \) of \textsuperscript{3}H-thymidine (specific activity 629 GBq/mM, obtained from BARC, India). The cell suspension was kept shaken at 120 rpm for 1 hr following which the cells were washed with chilled P-buffer and resuspended in 400 \( \mu l \) of the same buffer. 50 \( \mu l \) of the cell
suspension was directly added into scintillation vials containing 8 ml of Cocktail `W' (10 gm PPO, 0.25 gm POPOP and 100 gms napthalene in 1 liter of 1,4-dioxan) to monitor the uptake of labelled thymidine. Incorporation was determined by layering 50 µl of cell suspension on a 25 mm glass microfibre filter (Whatman) soaked with 25% TCA (w/v) solution and was washed three times with 10% TCA (w/v) solution. These filters were air dried, placed in a scintillation vial containing 8 ml of Cocktail `0' (6 gm PPO and 0.2 gm POPOP in one litre of toluene). The uptake and incorporation of labelled thymidine were determined in a Beckman scintillation counter.

7.2 Protein synthesis

Control and cisplatin treated Dictyostelium cells were resuspended in axenic medium at a density of 5x10^6 cells/ml. 300 µl of these growing cell suspensions were labelled at different time intervals (0, 6 and 12 hr after treatment) with 25 µCi/ml of 3H-leucine (specific activity 1850 GBq/mM obtained from BARC, India). The cell suspension was kept shaken at 120 rpm for 1 hr following which, the cells were washed in chilled P-buffer and resuspended in 300 µl of the same buffer. 75 µl of the cell suspension was directly added into scintillation vials containing Cocktail `W' to monitor the uptake of labelled thymidine. Incorporation was monitored by layering 75 µl of cell suspension on a 25 mm glass microfibre filter (Whatman)
soaked with 25% TCA (w/v) and was washed three times in 10% TCA (w/v) solution. These filters were air dried, placed in a scintillation vial containing 8 ml of cocktail "O". Radioactivity was measured in Beckman scintillation counter.
B. STUDIES ON MORPHOGENESIS

1. CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE (cAMP) CHEMOTAXIS

CAMP chemotaxis was monitored in control and cisplatin treated Dictyostelium cells.

1.1 Small-population assay

This assay was done according to the method of Ohmori and Maeda (1987). Briefly, control and cisplatin treated cells were harvested and suspended into thick slurry. 2 µl of this slurry was placed as a small droplet on agar containing 1 X 10^-5 M cAMP. The cells were scored positive when they moved out of the droplet.

1.2 cAMP dependent extracellular phosphodiesterase assay

The extracellular phosphodiesterase (ePDE) assay was done according to the method described by Riedel and Gerisch (1971). Control and cisplatin treated Dictyostelium cells were washed and resuspended in P-buffer at a density of 1 X 10^7 cells/ml. The cell suspensions were kept shaken at 100 rpm (22°C). 1 ml samples were withdrawn at 2 hr intervals from 4-12 hrs after the initiation of starvation. Cells were pelleted and supernatants were saved for ePDE assay.

Samples were assayed in 1 ml volume containing 0.1M Triethanolamine (TEA), (BDH), 0.4 Units of alkaline
phosphatase (Sigma), 0.25 Units of adenosine deaminase (Sigma), 50 nmoles of cAMP. To 900 μl of assay mixture, containing above ingredients (except cAMP), 50 μl of enzyme sample was added and incubated at 35°C for 10 min. 50 nmoles of cAMP (50 μl from 1μM cAMP stock) was added and the change in absorbance at 265nm was recorded using UV 2000 spectrophotometer (Hitachi) against a blank containing the complete assay mixture except cAMP. One ePDE Unit is defined as the quantity of enzyme which hydrolyses 1nmole of cAMP per minute at 35°C. ePDE activities of samples were calculated according to the following formula:

\[
\text{Change in absorbance} \times 100 \text{ nm/min/ml} \\
\text{Volume of sample} \times 8.1
\]

2. CHECK FOR EDTA STABLE CELL CONTACT FORMATION

Both control and cisplatin treated Dictyostelium cells were resuspended in P-buffer at a density of 1x10^7 cells/ml and placed on an orbital shaker (120 rpm) at 22°C. At regular intervals (4, 6, 8 and 10 hr, i.e., time from the initiation of starvation) 2 ml of samples were withdrawn and were kept in a 50 ml conical flask and EDTA was added to give a final concentration of 10 mM. The cell suspension containing EDTA was kept shaken for 1 hr at 80 rpm. The cell suspension was placed on a haemocytometer and the percent of loose cells was determined by single cell counts vs total cell counts (loose cells + cells forming
aggregates) (Ohmori and Maeda, 1987). The EDTA treated cell suspension was also put on a clean glass slide and photographed for record.

3. Morphogenesis on non-nutrient agar

Development of control and cisplatin treated Dictyostelium cells was monitored by plating the cells on non-nutrient agar. Dictyostelium cells were evenly spread on P-agar plates and were kept in an incubator at 22°C. At regular intervals development of Dictyostelium cells was monitored under a microscope and photographs of various stages were taken.
c. CYTOMORPHOLOGICAL STUDIES

1. LIVE CELL OBSERVATION

200 µl of cell suspension of control and cisplatin treated Dictyostelium cells were resuspended in fresh axenic medium in a multiwell test plate. The multiwell test plates were then observed under an inverted microscope (Axiovert-10, Carl Zeiss, Germany) and cells were photographed.

2. FIXED CELL PREPARATION

Control and cisplatin treated cell suspensions were spread on a clean subbed glass slides (prepared by immersing the slides in 2% gelatin and 0.1% chrome alum and then drying at room temperature). The cells were allowed to spread for 15min following which the extra fluid was drained off. The cells were then fixed with acetic acid:ethanol (1:3 v/v) for 10 minutes and stored in a air tight container till further processing.

2.1 Giemsa staining

Cells fixed by above procedure were stained with phosphate buffered 0.5% Giemsa solution, air dried and mounted in DPX. The slides were observed under a microscope (Aristophot, Leitz) and photographs were taken.
2.2 Toluidine blue staining

Equal volumes of 2% toluidine blue and 2% borax solutions were mixed to make 1% toluidine blue stain. Slides containing thin sections (1µm) of the control and treated cells were kept on a hot plate (35-40°C). Two drops of the stain was put on this slide for 10-15 sec. Then the slides were washed with distilled water, air dried and mounted in DPX. The slides were observed under a microscope (Aristophot, Leitz) and photographs were taken.

3. ELECTRON MICROSCOPY

Samples for electron microscopy were prepared according to the method described by Loomis (1975).

3.1 Scanning electron microscopy

Control and cisplatin treated Dictyostelium cells were kept shaken (100 rpm) in P-buffer for 10 hrs at 22°C following which they were fixed for 1 hr in chilled 2% glutaraldehyde (Sigma) made in 0.1M cacodylate buffer (pH 7.3). Cells were washed several times in chilled cacodylate buffer and post fixed for 1 hr in 1% Osmium tetraoxide (Sigma) made in 0.1M cacodylate buffer (pH 7.3). After final washing in chilled cacodylate buffer the cells were dehydrated in a graded series of ethanol, placed on a clean round glass and dried in a critical point drier. The specimens were coated with analytical grade silver in a
vaccum splutter. The specimens were observed in Philips SEM 501B, scanning electron microscope operated at 30 KV and photographs were taken.

3.2 Transmission electron microscopy

The control and cisplatin treated cells (cells treated for 1 hr were allowed to grow in fresh axenic medium for 12 hrs and cells in the continuous presence were taken after 24 hrs exposure to cisplatin) were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) for 1 hr, washed in the same buffer and post fixed for 1 hr in 1% Osmium tetraoxide made in 0.1M cacodylate buffer (pH 7.3), dehydrated in graded series of ethanol followed by propylene oxide and embedded in araldite. Thin sections (1 μm) were cut and stained with 1% toludine blue for light microscopic observation. Ultra thin sections ranging from 700-800 Å were cut, mounted on copper grids and stained with uranyl acetate and lead citrate. The grids were observed in a Philips CM10 transmission electron microscope operated at 70 KV and photographs were taken.