CHAPTER 2

Materials and methods

CHEMICALS

Hydroxylamine, cumene H_2O_2, benzamide, gallotannin, trypan blue, ALLN, NAD^+, agarose, Annexin V-FITC/PI dual staining kit, $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide), 2',7' dichlorodihydrofluorescein diacetate (H2DCFDA-AM), Protease Inhibitor Cocktail, anti-mouse IgG (whole molecule) FITC conjugate and anti rabbit IgG (whole molecule) were procured from Sigma. TRITC conjugate anti-PAR mouse mAb (10H) was procured from Calbiochem, Germany. Other reagents used in this study were of analytical grade and procured from Merck and Himedia.

METHODS

D. discoideum culturing conditions

D. discoideum, Ax-2 strain which is an axenic derivative of Raper's wild type NC-4 (a mutant in at least two genes i.e. *axe* A and *axe* B, Newell *et al.*, 1982 and Deering *et al.*, 1988) was used. *D. discoideum* was grown under different culture conditions. The growing cells (unicellular) were maintained in a liquid suspension (HL5 medium). *D. discoideum* cells were grown in modified HL-5 medium, pH 6.5 with 150 rpm shaking at 22°C (Watts and Ashworth, 1970). Log phase cells at a density of ~2.5 X 10^6 cells/ml were used for experiments.

It is also maintained on a solid substratum containing Phosphate Buffered Agar (PBA). *D. discoideum* was also cultured on bacterial lawn of *Klebsiella* which is its natural food. For this a loop full of overnight grown culture of *Klebsiella* is taken and *D. discoideum* spores (4-5) are mixed with it. This 'mixture' of two cell types is then pour plated on PBA plates (90mm). *D. discoideum* cells feed on *Klebsiella* and when no more *Klebsiella* is left the *D. discoideum* cells undergo developmental changes and form fruiting bodies.

Preparation of Axenic Medium (Oxoid) – (modified HL5medium, Ashworth and Watts, 1970; Kosta *et al.*, 2001)

Proteose peptone - 14.3 gm

Yeast extract - 7.5 gm

Maltose - 18 gm

$Na_2HPO_4\,2H_2O$ - 0.616 gm

$KH_2PO_4\,2H_2O$ - 0.486 gm

The above constituents were added to 1000ml of distilled water and pH was adjusted to 6.7. The medium (10 ml) was then dispensed into conical flasks and were autoclaved at 15 psi for 15 minutes. Care was taken so that the medium would not be charred by over heating.

Phosphate Buffer / Sorenson's buffer / KK_2 buffer (50X SB)

2mM Na_2HPO_4

15mM KH_2PO_4

pH 6.4

It was sterilized by autoclaving at 15 psi for 15 minutes. 1X SB buffer was obtained by adding 1 ml of 50X SB to 49 ml of autoclaved distilled water.

Phosphate Buffered Agar (PBA)

2 gm agar was added to 98 ml distilled water and autoclaved at 15psi for 15 minutes. After autoclaving 2 ml 50X SB was added.

Culture Maintenance

Growth of Ax-2 cells in Axenic medium (Levraud *et al.*, 2001)

i). 10 ml of the autoclaved HL-5 medium was taken and approximately 100 µl (inoculum size = 1% of medium volume, approx 0.05 X 10^5 cells/ml) of log

phase culture with a cell density of $2X10^6$ cells/ml were used for inoculation in the medium under sterile conditions.

ii). The flasks were incubated at 22 ^{0}C under shaking conditions maintained at a speed of 150 rpm.

iii). Subculturing was done every week. Subculturing can be done twice (till one month), after which the culture must be revived from the spores. The first or second subculture from this spore revival was used for experimental purposes.

Differentiation of *D. discoideum* cells on non nutrient agar (Levraud *et al.*, 2001)

i). Exponentially growing cells from HL-5 medium were pelleted down at 300g/5min/4^{0}C.

ii). The pellet was washed with 1X SB (Sorenson's buffer) thrice at 300g/5min/4^{0}C.

iii). The final cell density was adjusted to 2.5 X 10^6 cells / ml and 100µl of cell suspension was then spread on PBA (non-nutrient agar) containing 35 mm plate and allowed to differentiate at 22 ^{0}C to form fruiting bodies.

Spore Revival

- 5 ml HL-5 medium was dispensed in 25 ml conical flasks and autoclaved.
- The fruiting bodies were picked up from the surface PBA plates with a sterile Nichrome loop. The loop must not be allowed to touch the agar surface at any point, and all other precautions must also be taken to avoid bacterial contamination.
- Few fruiting bodies were inoculated in each flask, and then incubated under shaking conditions at 22 ^{0}C /150 rpm. Spores germinate with overnight incubation but log phase culture that can be used for further sub-culturing, could be obtained after about a week.

Cell viability test by trypan blue exclusion technique (Kosta *et al.*, 2001)

This is a vital staining procedure, where Trypan blue stains dead cells exclusively as live cells can pump out the dye. The stained dead cells then appear blue, while viable cells

remain colourless. 20 µl of the culture to be tested is taken and 10 µl of 0.1% Trypan blue solution is added. A cell count is taken using the haemocytometer. The percentage viability is then calculated using the following formula

Number of dead cells X 100 = % Dead cells

Total number of cells

Note: Trypan blue is commercially available as 0.4% stock solution. Diluted it 4 times with 1X SB to make 0.1% working solution.

Induction of oxidative stress by cumene H2O2 treatment

Oxidative stress was induced in *D. discoideum* cells by exogenous addition of cumene H2O2 (Sigma). Log phase cells at a density of ~2.5 X 10^6 cells/ml were exposed to cumene H_2O_2 (0, 0.04, 0.08 mM) in HL-5 medium at 22°C in a sterile flask. Cell viability was assayed using Trypan blue after 24 hours of cumene H2O2 stress (Kosta *et al.*, 2001).

Induction of oxidative stress by cadmium (Cd) (Sroka *et al.*, 2002)

2 X 10^6 cells were washed twice with Charkley's solution (pH 7.5). Then resuspended in 24 well plate having 1 ml Charkley's solution containig different concentration of cadmium (0, 0.1, 0.2, 0.5, 0.7mM) and incubate at 21^0 C for 1 hr. After incubation transfered the cells with ~~respected~~ respectively labeled ~~labled~~ tubes and washed atleast three times with 1x SB and then finaly resuspended in fresh HL5 medium. Cell viability was assayed using Trypan blue at every 2 hrs up to 24 hrs.

Charkley's solution (pH= 7.5)

14 mM NaCl

0.27 mM KCl

0.5 mM CaCl2

0.5 mM MgCL2

Assessment of cell death by AnnexinV-FITC/PI dual staining (Miller, 2004)

To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI was performed using Apoptosis detection kit (Molecular Probes, USA). Phosphatidyl serine (PS) exposure on the surface of the cells is one of the characteristic features of apoptotic cells. It is also a signal for macrophages to clear such cells by phagocytosis which is important to prevent inflammation and damage to the surrounding cells. PS has an affinity to bind to Annexin in a Ca^{+2} dependent manner. Annexin can bind to PS only when it is exposed outside as it cannot cross the membrane. Propidium Iodide (PI) is a DNA binding dye which can enter the cell only when membrane integrity is completely lost. Thus early apoptotic cells will be Annexin+ and PI- while late apoptotic and necrotic cells will be Annexin+ and PI+.

~$2.0X10^6$ cells were pelleted and washed twice with 1X SB. *D. discoideum* cells were then suspended in binding buffer provided in the kit and incubated with Annexin V and PI for 10 minutes in dark at 22°C. Fluorescence was monitored under 60X magnification using fluorescent microscope (Nikon Eclipse TE2000-S, Japan). Dose and time dependent study was done to standardize the paraptotic and necrotic doses for further experiments.

Measurement of PARP activity by indirect immunofluorescence (Cole and Perez-Polo, 2002)

PARP activity was assayed indirectly by using antibodies against the product of PARP i.e. PAR. For assaying PARP, indirect immunofluorescence was done using anti-PAR mouse mAb (10H) (Calbiochem, Germany) at a concentration of 0.5ug/ml and anti-mouse IgG (whole molecule) FITC conjugate (Sigma) at a dilution of 1:200. *D. discoideum* cells were pelleted and washed once with phosphate buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 minutes at -20 °C and then washed with blocking solution (1.5% BSA with 0.05% Tween 20 in PBS) followed by incubation for 1 hour in primary antibody. After incubation the cells were washed 2-3 times with blocking solution and further incubated for 1 hour with FITC labeled secondary antibody. Finally these cells were washed 2-3 times with PBS and the fluorescence was observed at 490 nm under 60X magnification. PARP assay was performed at different time intervals as described in the experiments later.

ROS measurement by DCFDA dye (Esposti, 2002)

In order to observe the formation of reactive oxygen species a fluorescent dye is employed. The acetate ester form of 2',7' dichlorodihydrofluorescein diacetate (H2DCFDA-AM) is a membrane permeable molecule that passes through the cell membrane.

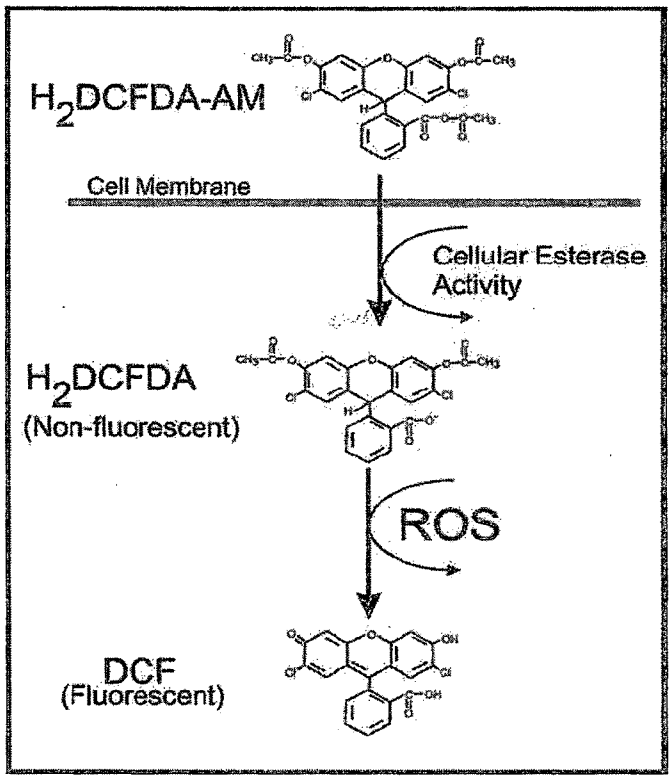

Figure 2.1 Formation of fluorescent Compound DCF by ROS

Once inside the cell, cellular esterases act on the molecule to form the non-fluorescent moiety H2DCFDA, which is ionic in nature and therefore trapped inside the cell. Oxidation of H2DCFDA by ROS converts the molecule to 2',7' dichlorodihydrofluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant production of ROS causes an increase in fluorescence signal over time.

$2X10^6$ cells were harvested and washed with 1X SB twice. DCFDA (50 nM) was added to cells and was incubated for 15 min at 22 ^{0}C with shaking, followed by two washes with 1X SB. Fluorescence unit was measured by fluorimeter (F7000, Hitachi, Japan) using 200 µl sample diluted 5 times using KK2 buffer. Excitation (λex) and emission (λem) wavelengths used for fluorimetric studies were is 480 and is 525 nm respectively.

DNA damage (Minami *et al.*, 2005)

Immunofluorescence was done using anti-γH2AX mouse antibody (kind gift from Dr. Rekha Rai) at 1:500 dilution and anti-mouse IgG (whole molecule) TRITC conjugate (Sigma) at a dilution of 1:200. *D. discoideum* cells were pelleted and washed once with phosphate buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 minutes at -20 °C and then washed with blocking solution (1.5% BSA with 0.05% Tween 20 in PBS) followed by incubation for 1 hour in primary antibody. After incubation the cells were washed 2-3 times with blocking solution and further incubated for 1 hour with TRITC labeled secondary antibody. Finally these cells were washed 2-3 times with PBS and the fluorescence was observed under 60X magnification.

Estimation of NAD^+ (Bernofsky and Swan, 1973)

Intracellular levels of NAD^+ in *D. discoideum* were determined by enzymatic recycling method employing alcohol dehydrogenase to reduce NAD^+ to NADH. NAD^+ present in the cell is used to convert ethanol into acetaldehyde, the reaction being catalyzed by alcohol dehydrogenase. NADH produced in the first reaction is used to reduce MTT in to Formazan and NAD^+ is regenerated. In this way cellular NAD^+ keeps on getting recycled and thereby enhances the yield of Formazan, a chromophore which gives absorbance at 570 nm. In brief, cells were exposed to 0.03 and 0.05 mM cumene H_2O_2 for 1 hour and cultures were washed twice with ice cold PBS and NAD^+ was extracted with 1 ml of 0.5 M perchloric acid and then neutralized with 1 N KOH. NAD^+ levels were estimated by taking the absorbance at 570 nm following protein estimation with Lowry method (Lowry *et al.*, 1951).

Evaluation of mitochondrial membrane potential (MMP) (Koning *et al.*, 1993)

Potential sensitive dye $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide) (Sigma) was used to evaluate changes in mitochondrial membrane potential (MMP). $DiOC_6$ is a membrane permeable lipophilic cationic dye which binds specifically to mitochondria proportionally to the difference between the negativity of the cytoplasmic compartment and the mitochondrial matrix. Dissipation of the mitochondrial membrane potential reduces the affinity of binding of the dye.

To observe the change in MMP, time dependent study was done using 0.03 mM cumene H_2O_2 dose (paraptotic dose) and 0.05 mM cumene H_2O_2 dose (necrotic dose) as standardized by PS-PI dual staining. ~$2.0X10^6$ cells were pelleted and washed twice with 1X SB. Cells were stained with $DiOC_6$ (400 nM) for 15 minutes in dark and then washed once with 1X SB and monitored for the fluorescence as above.

PARP inhibition by benzamide or gallotannin (Szabo and Dawson, 1998)

Log phase culture of *D. discoideum* with a cell count of $2X10^6$ cells was incubated with different doses (1.0, 2.0, 3.0, 4.0 mM) of benzamide (Sigma, USA) or (0, 1, 10, 20mM) of NAD^+ (SRL, India) for 12 hours. For further experiments, 1 mM benzamide and 10mM NAD^+ was used as it showed 2% and 3% cell death respectively. Cells were preincubated with 1 mM benzamide and then treated with HA or cumene H_2O_2 and observed for different cell death parameters. To show the specificity of benzamide similar studies were also carried out with non inhibitory structural analog of benzamide, benzoic acid (Jyotika, 2009).

Investigation of proteases involved in cell death (Moubarak *et al.*, 2007)

Cells were pre-incubated with protease inhibitor cocktail (Sigma, USA) followed by HA stress and MMP changes and PS exposure were checked. Similarly MMP changes, PS exposure and vesicle formation were followed in H2O2 or cadmium treated cells pre-incubated for 2 hours with 50 μM ALLN, a Calpain inhibitor.

Plasmid isolation from *E. coli*

Alkaline lysis method for isolation of plasmid from *E. coli* was followed (Sambrook and Russell, 2001).

Transformation of plasmid DNA in *E.coli* This was performed as per (Sambrook and Russell, 2001)

Transformation of plasmid DNA in *D. discoideum* (Gaudet *et al.*, 2007)

$5x10^6$ cells were pelleted down. Washed twice with ice cold H-50 buffer. Added 2 µl (1-10 µg DNA) of cloned plasmid DNA. Incubated on ice for 5 minutes. The cell suspension was transferred to a cold 0.2 cm electroporation cuvette. Electroporated in cold at 0.8kV/25 µF pulse, twice for about 5 second gap between 2 pulses. Incubated the cuvette on ice for 5 minutes. Set up 10cm Petri dish for selection with sterile 10ml HL5 and cells were transferred out of cuvette with a few ml of HL5 to the plate. Incubated in BOD at 22 °C for overnight.

H-50 buffer

4.76 gm HEPES

3.73 gm KCl

0.58 gm NaCl

0.12 gm $MgSO_4$

0.12 gm $NaHCO_3$

0.156 gm NaH_2PO_4

700 ml D/W (pH 7.0 with HCl /NaOH) and made up to 1000 ml final vol.
Autoclaved and kept at 4^0 C.

E-buffer

10 mM $NaPO_4$ (pH 6.1) in 50 mM sucrose. Autoclaved and kept at 4^0 C.

Added antibiotics from the next day with medium change as follows:

	1st day	3rd day	5th day	Alternate day
Ampicillin	1µg/ml	1 µg/ml	1 µg/ml	1 µg/ml
G418	5 µg/ml	10 µg/ml	15 µg/ml	15 µg/ml

Developmental studies

Effect of GSH/ benzamide on cadmium induced *D. discoideum* development

D. discoideum cells were subjected to 1 mM GSH treatment for 1 hour, then subjected to 1 hour Cd treatment (0, 0.2, 0.5mM) without removing the glutathione. Cells were then washed and the pellet was resuspended in 100µl of Sorenson's buffer (1X SB) and spread on PBA plate (Sroka *et al.*, 2002).

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