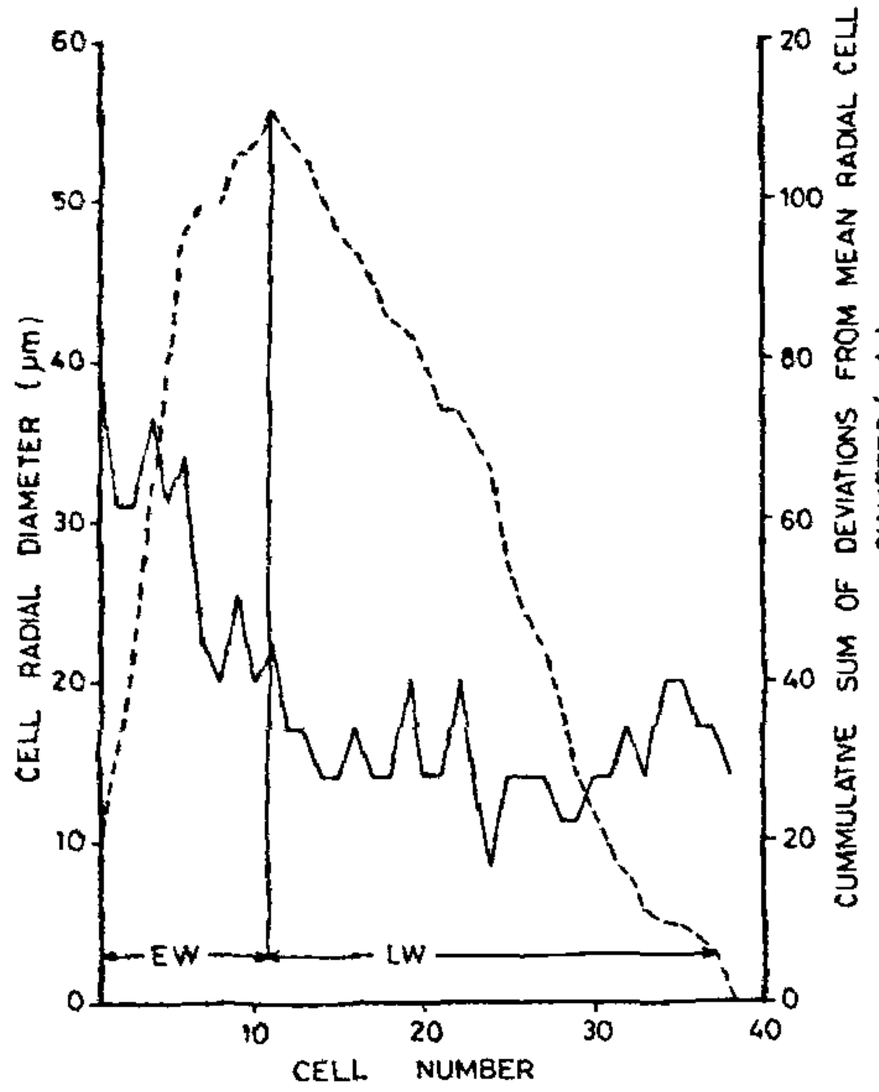

Figure 2. Cell radial diameter changes across a ring in *S andrewsii* Bose *et* Sah Points from where the cumulative sum of deviations turn sharply towards zero indicates the earlywood/latewood boundary. EW, earlywood; LW, latewood.

little amount of early wood in comparison to latewood. This type of curve (Figure 2) is characteristic of 'A' type after Creber and Chaloner[4]. It has been shown earlier that the development of earlywood largely depends on the stored food reserves, however, the latewood is dependent on the current years assimilates which are available throughout the growing season[19]. The presence of large amount of latewood in the fossil wood of *Sahnioxylon* shows the extended growing season with favourable conditions when this tree flourished in the Rajmahal area during the early Cretaceous.

Based on palaeofloral evidences from Rajmahal Formation in Rajmahal Hills, Bihar a sub-tropical to tropical type of climate has been suggested[17]. However, the growth ring features of *Sahnioxylon* (*S. rajmahalense* and *S. andrewsii*), especially very wide rings with large amount of latewood, are indicative of a sub-tropical to warm temperate climate.

1. Fritts, H. C, *Tree Rings and Climate*, Academic Press, London, 1976.
2. Schweingruber, H, *Tree Rings· Basics and Applications of Dendrochronology*, Kluwer, Netherlands, 1988.
3. Chaloner, W. G. and Creber, G. T., *Implications of Continental Drift to the Earth Sciences* (eds Tarling, G T. and Runcorn, S. K), Academic Press, London, 1973, pp 425–437.
4. Creber, G. T. and Chaloner, W. G, *Bot Rev*, 1984, 50, 357–448.
5. Creber, G. T. and Chaloner, W. G., *Palaeogeogr. Palaeoclimatol Palaeoecol.*, 1985, 52, 35–60.
6. Jefferson, T. H., *Palaeontology*, 1982, 25, 681–708.
7. Jefferson, T. H, *Antarctic Earth Sciences* (eds Oliver, R L., James, P. R and Jago, J B), Australian Academy of Sciences, Canberra, 1983, pp. 593–598.
8. Francis, J. E, *Palaeogeogr. Palaeoclimatol. Palaeoecol*, 1984, 48, 285–307.
9. Francis, J. E., *Palaeontology*, 1986, 29, 665–684.
10. Ash, S. R. and Creber, G. T., *Palaeogeogr. Palaeoclimatol. Palaeoecol.*, 1992, 96, 299–317.
11. Sahni, B, *Mem. Geol. Surv. India, Palaeont. Indica* (n.s), 1932, 20, 1–19.
12. Hsu, J. and Bose, M. N., *J. Indian Bot. Soc.*, 1952, 31, 1–12.
13. Bose, M. N. and Sah, S C. D., *Palaeobotanist*, 1954, 3, 1–8
14. McDougall, I. and McElhinny, M. W., *Earth Planet. Sci. Lett.*, 1970, 9, 371–378.
15. Maheshwari, H. K. and Jana, B. N., Proceedings of the symposium on Cretaceous of India: Palaeoecology, Palaeogeography and Time Boundaries (ed. Maheshwari, H. K.), Indian Assoc. Palynostratigraphers, Lucknow, 1983, pp. 158–192.
16. Singh, H. P. and Venkatachala, B. S., *Palaeobotanist*, 1987, 36, 168–176.
17. Sukh-dev, *Palaeobotanist*, 1987, 36, 161–167
18. LaMarche, V. C. *Science*, 1974, 183, 1043–1048.
19. Larson, P. R., *Silvae Genet.*, 1967, 10, 58–65

Received 21 March 1994; revised accepted 4 August 1994

Growth-phase-dependent binding of hematoporphyrin derivative to human glioblastoma (U-87MG) cells

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Studies on the binding of hematoporphyrin derivative (HpD) to asynchronous and synchronized human glioblastoma (U-87MG) cells were performed using fluorescence spectroscopy. Cell-bound HpD exhibited three fluorescence bands. The intensity distribution of fluorescence spectrum was found to be dependent on the growth phase of cells. The relative intensity of 636 nm band increased as the cells progressed through the growth curve. A more distinct change was observed when the fluorescence spectrum of Hpd bound to M-phase cells was compared with G1-phase cells. M-phase cells exhibited maximum fluorescence emission at 615 nm, with a small shoulder at 636 nm whereas in G1-phase cells the 636 nm fluorescence band showed enhancement. HpD binding to synchronized cells was also studied during the cell division cycle. A clear variation in the intensity ratio of 636 to 615 nm fluorescence bands was observed along the cell division cycle. This intensity ratio was maximum in mid G1 and early G2 phases of growth, whereas a minimum was observed in mid S phase. The photosensitivity of asynchronous as well as synchronized cells was determined under above-mentioned experimental conditions. The photosensitivity also varied with the growth phase of the cells. A good

correlation was found between the photosensitivity and the fluorescence intensity distribution of cell-bound HpD.

PHOTOSENSITIZER hematoporphyrin derivative (HpD), which is retained preferentially in malignant tissue compared to some normal tissues, has shown promising results in photodynamic therapy of tumours[1–5]. In order to understand the photodynamic action of HpD, drug localization and photosensitization have been investigated in different types of cells[6–14]. The photodynamic action of HpD has also been investigated in synchronized CHO and NHIK 3025 cells[8, 12]. In NHIK 3025 cells, a large variation in the photosensitivity along the cell cycle was found, whereas CHO cells did not show any change in photosensitivity along the cell cycle. Previously, we have studied HpD binding and photosensitivity in brain tumour (BMG-1) cells. The fluorescence spectra of cell-bound HpD varied under different experimental conditions and a good correlation was found between the photosensitivity and the fluorescence spectral distribution[10, 11]. The present paper deals with the binding of HpD to asynchronous and synchronized human glioblastoma (U-87MG) cells and their photosensitivity. Our results demonstrate that HpD binding and photosensitivity are highly dependent on the growth phase of the cells.

Hematoporphyrin dihydrochloride (Hp), ethidium bromide and RNase were obtained from Sigma Chemical Company, St. Louis (USA). Eagles minimum essential medium (EMEM), phosphate-buffered saline (PBS) and pepsin were purchased from Hi Media, Bombay (India). Foetal calf serum (FCS) was procured from Northumbria Biochemicals Ltd., London (UK). All other chemicals were analytical grade. Hematoporphyrin derivative (HpD) was prepared from Hp as described earlier[9].

Human glioblastoma cell line (U-87MG) was obtained from American Type Culture Collection (ATCC), Rockville (USA). Cells were grown in Nunc plastic tissue culture flasks. The growth medium was EMEM supplemented with 5% FCS, 5% bovine serum (BS), 1 mM sodium pyruvate and antibiotics. Synchronized cells were obtained by mitotic selection without using any mitotic inhibitor[15]. 1.5×10^6 cells were plated in 25 cm^2 plastic culture flasks. After 24 h of growth, loosely attached cells were removed by shaking the flask at 400 rpm for 5 min in a rotatory shaker. Cells detached in the first shake were discarded. Attached cells were replenished with prewarmed fresh growth medium and incubated for 40 min at 37°C. The shaking procedure was repeated and mitotic cells were collected from the medium by centrifugation. Mitotic cells simultaneously obtained from 8–10 bottles were pooled and seeded in 20 cm^2 culture dishes (0.1×10^6 cells per dish). The growth of synchronized cells was monitored by counting the cells at different time periods in a specified area marked in the dish. At different time points cells were treated with HpD and fluorescence spectra were measured using SLM 8000C spectrofluorometer as described earlier[11].

Hpd treatment was performed at different time points by incubating the cells in monolayers with desired amount of HpD for 15 min at 37°C. Subsequent to HpD treatment the cells were washed three times with PBS to remove the free dye. Cells were then released using a rubber policeman and suspended in PBS (pH 7.4) at a concentration of approximately 1×10^6 cells/ml.

The distribution of cells in different phases of growth was determined by measuring the DNA content in single cells by flow cytometry. At different times after plating, cells were detached from the culture dishes using rubber policeman and were fixed in ice-cold 90% ethanol. Fixed samples were centrifuged at 1000 rpm for 10 min and cells in the pellet were washed with PBS (pH 7.4). Washed cells were treated with pepsin (0.5%) for 1 h at 37°C. Cells were washed again and incubated with PBS containing 25 µg/ml ethidium bromide, 40 µg/ml RNase and 0.3% nonidet P-40 for 20 min at room temperature. Fluorescence from cellular DNA labelled with ethidium bromide was measured by Becton Dickinson Facscan. Excitation was performed at 488 nm and fluorescence emission was collected using a 520 nm long-pass filter.

Photosensitivity was measured by the ability of cells to form macroscopic colonies[11]. After HpD treatment, cells were irradiated by placing the petri dishes directly below two cool daylight fluorescent tubes (40 W each) covered with a perspex diffuser sheet. The emission of this source was in the range 400–700 nm and the fluence rate was about 1 W/m^2. After irradiation, the medium containing HpD was removed and growth medium was added. Cells were then incubated at 37°C for 7 days to form macroscopically visible colonies. Colonies containing more than 50 cells were scored. Each experiment included control cultures treated with HpD but having received no light irradiation. For experiments on asynchronous cultures 0.35×10^6 cells were plated in 25 cm^2 Nunc plastic tissue culture flasks. At 24 h interval, cells were released from different flasks. HpD binding and photosensitivity were studied as described above.

The binding of HpD to asynchrous U-87MG cells was studied along the growth curve and the data are shown in Figure 1. Cell-bound HpD exhibited three fluorescence bands at 615, 636 and 678 nm, but their intensity distribution varied with the growth of cells. The relative intensity of the 636 nm fluorescence band gradually increased as the cells progressed along the growth curve and the intensity was maximum for a confluent culture. The photosensitivity of cells, studied under similar conditions, was also found to be dependent on the growth of cells. An increase in the photosensitivity was observed as the cells progressed towards the con-

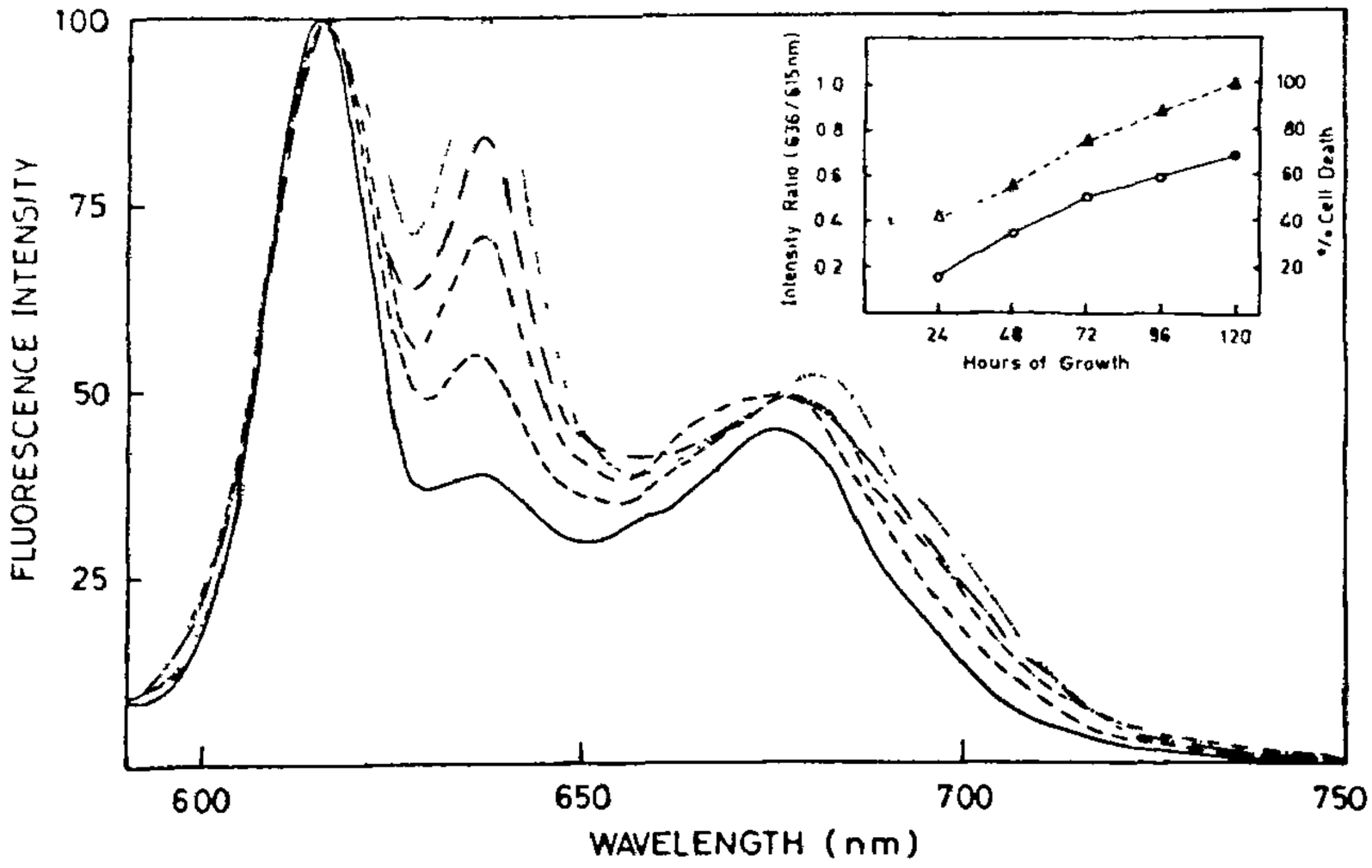

Figure 1. Fluorescence spectra of HpD bound to U-87MG cells at different times along the growth curve: —— 24 h, – – – 48 h, –·–·– 72 h; – – –·– 96 h; ..... 120 h. Inset –Δ–Δ– Fluorescence intensity ratio (636/615); –o–o– percentage cell death along the growth curve. Cells were incubated for 15 min with 25 μg/ml HpD in the presence of 10% serum.

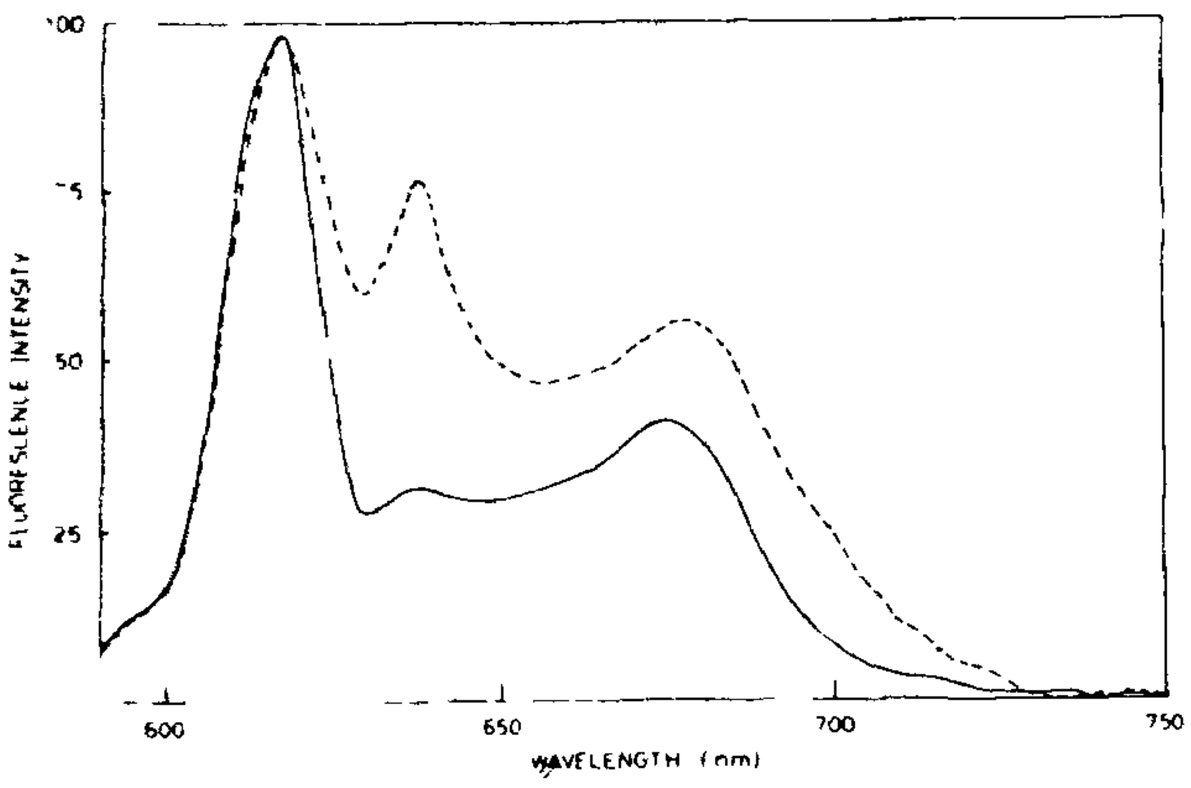

Figure 2. Fluorescence spectra of HpD bound to M-phase (——) and G1-phase (– – –) U-87MG cells. Cells were incubated for 15 min with 25 μg/ml HpD in the presence of 10% serum.

fluence. The photosensitivity and ratio of the fluorescence intensity at 636 and 615 nm in the spectra of cell-bound HpD as a function of cell growth is depicted in the inset of Figure 1. As the relative fluorescence intensity of the 636 nm band increased with cell growth, an increase was observed in cell death after light irradiation.

In asynchronous culture the populations of cells in different phases of growth (G1, S, G2 and M) vary as the culture grows. It is likely that cells in different phases of growth have different binding properties which give rise to the observed changes in the fluorescence spectra along the growth curve. Figure 2 shows the fluorescence spectra of HpD bound to synchronized M-phase cells isolated by mitotic selection. HpD spectrum observed in G1-phase cells is also shown for comparison. The cells were incubated with HpD for 15 min to ensure that M-phase cells do not enter into the G1 phase. Interestingly, the fluorescence spectra of HpD bound to M-phase cells showed a prominent 615 nm band, with low fluorescence intensity at 636 nm. On the other hand, the spectra of HpD bound to G1-phase cells showed increased intensity at 636 nm. From these data it is clear that the binding of HpD to M-phase cells is different from that to cells in the G1 phase of growth.

We have further investigated the HpD binding and photosensitivity of synchronized cells as they progressed through the cell division cycle. Synchronized M-phase cells were obtained by mitotic selection and were grown further. At different intervals after synchronization, these cells were treated with HpD for 15 min and fluorescence spectra of cell-bound HpD recorded; under similar conditions, the photosensitivity was also measured. Figure 3 shows the fluorescence intensity ratio of 636 to 615 nm bands, photosensitivity of cells and the growth curve for synchronized cells. The ratio of fluorescence intensity at 636 nm to that at 615 nm (panel A) showed a clear variation with time and was maximum at about 5 h and 12 h after synchronization. A similar variation in cell death was observed along the cell division cycle as shown in panel B. The growth of synchronized cells was also monitored. As depicted in panel C, cells started dividing after about 12 h of mitotic selection and most of the cells divided during 12–16 h after synchronization.

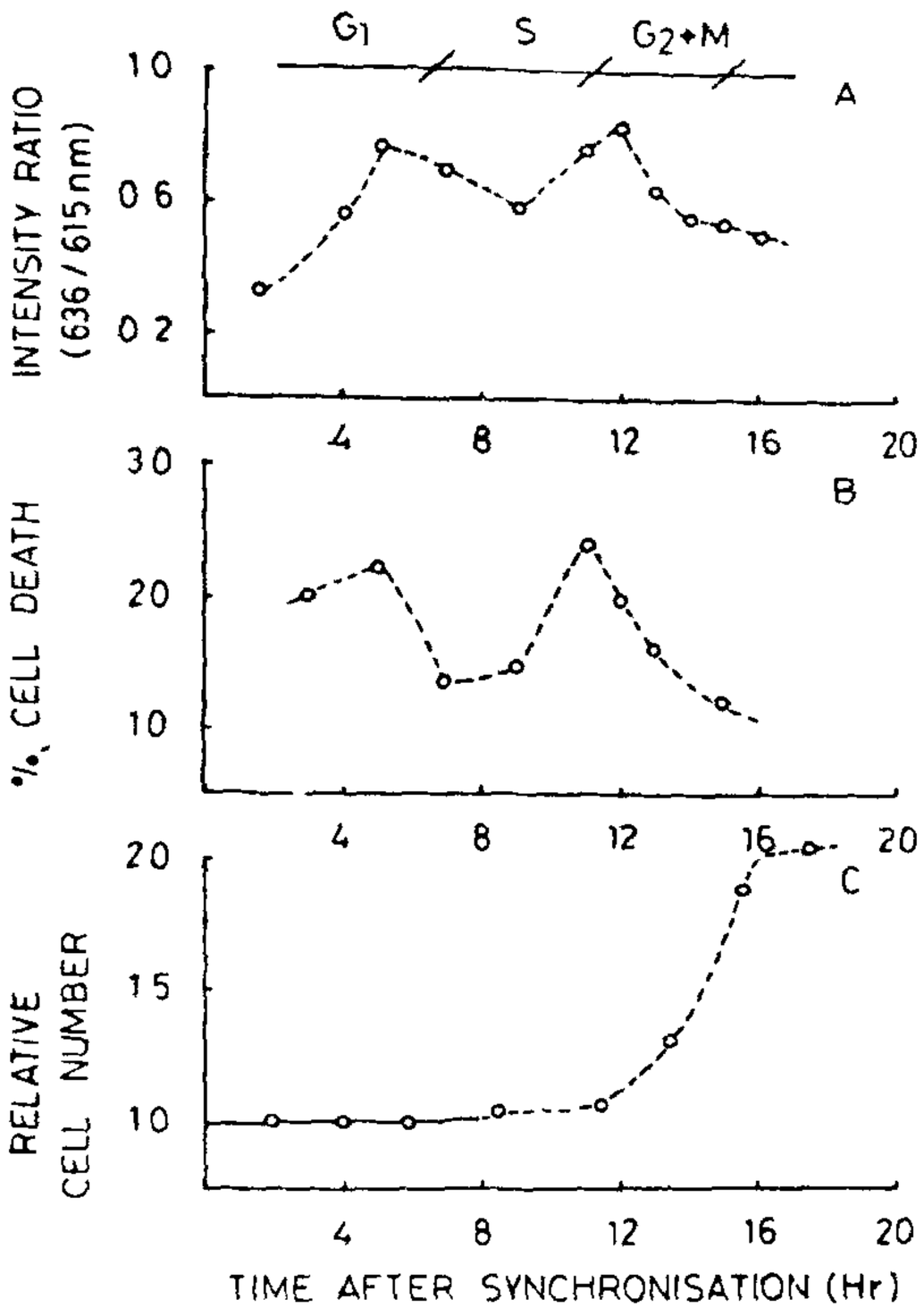

Figure 3. **Panel A.** Ratio of the 636 to 615 nm fluorescence intensities of HpD bound to U-87MG cells at different times after synchronization. **Panel B.** Percentage cell death at different times after synchronization. **Panel C.** Growth curve for synchronized U-87MG cells. The duration of G1, S and G2 + M phases was determined using flow cytometry. Cells were incubated for 15 min with 25 µg/ml HpD in the presence of 10% serum.

Distribution of cells in different phases along the cell cycle was obtained by flow cytometric measurement of DNA content in single cells. The duration of different phases of cell cycle in U-87MG cells was estimated from cell cycle distribution[15] using a doubling time of 16 h. The durations of G1, S and G2 + M phases were found to be 6.8 ± 1 h, 4.9 ± 0.6 h and 3.3 ± 0.9 h, respectively. It is clear from Figure 3 that the fluorescence intensity of the 636 nm band is maximum when the cells are in mid G1 phase or early G2 + M phase, whereas it is minimum in mid-S-phase cells. Since M-phase cells exhibit the fluorescence maximum at 615 nm (Figure 2), the second maximum in Figure 3 (panel A) corresponds to cells in the G2 phase.

The results presented here show that the fluorescence intensity distribution of cell-bound HpD depends on the growth phase of cells and the photosensitivity of cells is associated with the increased fluorescence intensity of cell-bound HpD at 636 nm. Previously, we have shown[11] that the fluorescence bands at 615 and 636 nm originate from different cellular sites which preferentially bind monomeric and oligomeric components of HpD, respectively[11]. The fluorescence band of HpD at 636 nm is observed in non-polar solvents, whereas in polar solvents HpD exhibits the 615 nm band. On the basis of this, we suggest that the origin of the 615 and 636 nm fluorescence bands in the cells may be due to the binding of HpD to some hydrophilic and hydrophobic sites, respectively. In the present study, the binding was measured after incubating the cells with HpD for 15 min under these conditions, HpD presumably binds to the plasma membrane of the cells[16]. We conclude that the observed changes in fluorescence spectra are due to alterations in the properties of cell membranes during the cycle of cell division. It has been suggested[17] that a number of cell surface and cytoplasmic molecules are expressed at varying levels during the cell cycle. Although the identity of the molecules binding to HpD is not yet clear, there are indications[18] that HpD binds to proteins in the cells. It is possible that the HpD binding proteins are expressed at different levels in different phases of growth. The cell surface changes occurring during the cell cycle may alter the properties of the cell membrane. Changes in cell membrane fluidity during the cell cycle have been shown earlier[19]. It is likely that in U-87MG cells also the difference in plasma membrane properties in different phases of growth may be responsible for the observed dependence of HpD binding to cells during the cell cycle.

1 Dougherty, T. J, *Photochem. Photobiol*, 1993, **45**, 895–900.
2 Moan, J. and Berg, K., *Photochem. Photobiol.*, 1992, **55**, 931–948.
3. Kaye, A H., Morstyn, G. and Appuzzo, M. L. J., *J. Neurosurg.*, 1988, **69**, 1–14.
4 Spikes, J. D and Jori, G, *Lasers Med. Sci.*, 1987, **2(3)**, 3–15.
5 Henderson, B. W. and Dougherty, T. J., *Photochem. Photobiol.*, 1992, **55**, 145–157.
6. Christensen, T, Sandquis, T, Feven, K., Waksvik, H and Moan, J., *Br. J. Cancer*, 1983, **48**, 35–43.
7. Jori, G. and Spikes, J. D., in *Topics in Photomedicines* (ed Smith, K. C.), Plenum, New York, 1984, pp. 183–318.
8. Gomer, C. J and Smith, D. M, *Photochem. Photobiol.*, 1980, **32**, 341–348.
9. Gangola, P. and Joshi, N. B., *Indian J. Med Res. (B)*, 1990, **92**, 467–470.
10. Joshi, K., Joshi, P. G and Joshi, N. B., *Curr. Sci.*, 1991, **61**, 413–414.
11. Joshi, K, Joshi, P. G. and Joshi, N. B, *Photochem. Photobiol.*, 1992, **55**, 113–118.
12. Christensen, T., Feren, K., Moan, J. and Pettersen, E, *Br. J. Cancer*, 1981, **44**, 717–724.
13. West, C. M L., West, D. C., Kumar, S. and Moore, J. V., *Int. J. Radiat. Biol.*, 1990, **58**, 145–156
14. Shulok, J. R., Wade, M. H and Lin, C. W., *Photochem Photobiol*, 1990, **54**, 451–457.
15 Pettersen, E. O, Bakke, O, Lindmo, T. and Oftebro, R., *Cell Tissue Kinet.*, 1977, **10**, 511–522.
16. Kessel, D, *Photochem. Photobiol*, 1986, **44**, 489–493
17. Hall, P. A. and Woods, A. L., *Cell Tissue Kinet.*, 1990, **23**, 505–552.
18. Moan, J. and Vistnes, A. I, *Photochem Photobiol*, 1986, **44**, 15–19

19 De Latt, S W, Vander Saag, P T and Shmitzky, M, *Proc Natl. Acad Sci*, 1977, 74, 4458–4461

ACKNOWLEDGEMENTS We thank Prof Moudgal, IISc, Bangalore for the use of Flow Cytometer This work was supported by Department of Science and Technology, Govt. of India, New Delhi

Received 28 April 1994, revised accepted 22 July 1994

Production of intergeneric hybrid between *Oryza sativa* L. and *Porteresia coarctata* T.

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Intergeneric hybrids were produced by wide hybridization between salt-sensitive rice cultivars (IR 28 and IR 36) and a salt-tolerant perennial wild grass species *Porteresia coarctata* ($2n = 4x = 48$). *In vitro* excision of 6 days old fertilized ovule could rescue eighteen globular, undifferentiated hybrid embryos. Germination of such embryos on quarter strength MS medium without hormone produced 10 viable hybrid plants. The crossability ranged from 0.09 to 0.13%. The hybrids were short and possessed phenotypic characters like light purple basal leaf sheath, short ligule and short awn resembling *P. coarctata*. Meiotic chromosome analysis revealed triploid nature of the hybrids with 36 chromosome number. The hybrids were completely male sterile.

THE genus *Oryza* has two cultivated species – *Oryza sativa*, *O. glaberrima* and about 18 wild species. The cultivated rice species share a common genome AA with its closely related wild species and thus gene transfer is achieved without much difficulty[1]. The distantly related wild species of *Oryza* have genomes which have been designated as BB, BBCC, CC, CCDD, EE and FF. The transfer of genes of economic importance has been possible across crossability and recombination barriers from CC, BBCC and EE genomes into AA genome of cultivated rice[2–4]. However, cultivated rice germplasm as well as wild species of the genus *Oryza* do not possess genes for tolerance to abiotic stresses like salinity and limit rice production in coastal rice growing areas of tropical countries[5,6].

The wild perennial grass species *Porteresia coarctata* Tateoka, formerly a member of the genus *Oryza*[7] and *Sclerophyllum*[8] grows in the coastal salt-marshes of Bangladesh and India. The species is distinct from the species of *Oryza* morphologically and anatomically and is a tetraploid with 48 chromosomes[9]. This wild grass species has been considered as a rich source of genetic material for transfer of salt tolerance genes into *O. sativa*[10]. However, it has not yet been successfully hybridized with *O. sativa* because of high degree of crossability barriers between the two genera[11]. In order to increase salt tolerance of cultivated rice through transfer of salt tolerance genes, we report here the successful production of an intergeneric hybrid between *O. sativa* and *P. coarctata*.

Three high-yielding rice cultivars such as IR28, IR36 and Tellahamsa which are sensitive to soil salinity were used as female parents in this study. The seeds of IR28 and IR36 were obtained from the International Rice Research Institute (IRRI), Manila, Philippines and Tellahamsa seed was obtained from the Directorate Rice Research (DRR), Rajendranagar, Hyderabad. Young tillers of *P. coarctata* were obtained from the Central Rice Research Institute (CRRI), Cuttack, Orissa which were originally collected from Bhittar Kanika seashore of Bay of Bengal, Orissa. The tillers of *P. coarctata* were multiplied in several pots and used as male parent in all cross combinations. Three rice varieties were also planted on different dates in the greenhouse and were used as female parents in crosses with *P. coarctata*.

The spikelets of three rice cultivars were emasculated before anthesis in the evening hours and pollinated heavily with pollen of *P. coarctata* at the same time. The pollinated spikelets were sprayed with 75 ppm GA3 (giberellic acid) after 12–24 hours of pollination for 5 days. On the 6th day, pollinated spikelets were collected and examined for the development of fertilized ovule aseptically. The immature embryos were excised and cultured on quarter strength MS medium devoid of hormones according to the method of Jena and Khush[12]. The cultured embryos showed delayed germination and germinated seedlings at two leaf stage were transferred to Yoshida's modified Hogland solution in small pots and allowed to grow until maturity. The crossability was determined according to the formulae of Wu *et al*[13] and morphological traits of the hybrids were recorded. Cytological analysis of the hybrids was done at diakinesis and metaphase I stages of meiosis. For cytological analysis, spikes of the hybrids at a suitable stage were fixed in a fixative containing acetic acid and ethanol (1 : 3, v/v) to which traces of ferric chloride was added. Meiotic analysis was done with 1% acetocarmine squash technique.

Highly salt sensitive rice cultivars, IR28, IR36 and Tellahamsa were used as female parents in crosses with *P. coarctata*. Of the 2160 spikelets of IR28 pollinated with *P. coarctata* pollen, only 0.46% spikelets had embryo development and four immature embryos could be excised aseptically and two plants were obtained out of three germinated embryos on quarter strength MS medium. In the cross between IR36 and *P. coarctata*, very low frequency of embryo development (0.30%) was obtained but eight plants could be germinated after