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Cytogenetic Testing of Mutagenic Potential of the Blue-Green Bacterium, *Plectonema boryanum* in Experimentally Treated Mice

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Microbes are submicroscopic and microscopic organisms including viruses, prokaryotic bacteria and eukaryotic fungi, algae and protozoa (Stanier *et al.* 1976). The genotoxic potential of animal viruses in man and mammals has been reported mainly by workers outside India (Bartsch 1970, Manna 1980), while similar potential of nearly 2 dozen species of bacteria, about a dozen of fungi, 4 species of parasitic protozoans and 1 species of unicellular alga has almost exclusively been documented by Manna and his collaborators using one or more mutagenicity testing protocols assessed generally in experimentally treated mice (Manna 1992, 1993) and some on fish (Manna and Sadhukhan 1992, Manna and Biswas 1992a, b). The bacteria and blue-green algae (Cyanophyceae) were used to be put into separate groups under prokaryotes but now blue-green algae are included in blue-green bacteria (Stanier *et al.* 1976, Vashistha 1986). Out of 200 odd genera listed under 17 sections in Bergey's Manual of Systematic Bacteriology (Krieg 1984, Sneath 1986), the mutagenic potential of some 2 dozen species of bacteria belonging to 18 genera and 10 sections was documented but none belonged to blue-green bacteria for which the present study was undertaken. Some Cyanophytes produce toxins causing mortality to fish and other animals (Munro 1978). Anyhow the present study has filled up a lacuna and substantiated more towards 'Microbes as Living Mutagens' advocated from time to time by Manna (1973, 1980, 1989, 1992, 1993).

Materials and methods

Laboratory bred Swiss albino mice, *Mus musculus* weighing between 20 and 25 g were used as experimental model for various advantages (Manna 1991) to test the mutagenic potential of various samples of the blue-green bacterium, *Plectonema boryanum* against parallel controls. *P. boryanum* (Scytonemataceae, Scytonematales, Cyanophyceae, Cyanophyta) is a filamentous, non-heterocystous and non-nitrogen fixing strain, sensitive to LPP-1 cyanophage and reproduces by means of hormogonia. The pure culture of *P. boryanum* was kindly supplied by Dr. D. N. Tiwari which was subcultured in modified Chu-10 medium containing mg/l: $Ca(NO_3)_2 \cdot 4H_2O$, 57.63; $MgSO_4 \cdot 7H_2O$, 25; $NaHCO_3$, 15.85; Na_2SIO_3, 10.87; $CaCl_2 \cdot 2H_2O$, 35.8; KH_2PO_4, 7.8; FeEDTA, 0.5 and the following micronutrients (μg/l): H_3BO_3, 0.715; $MuCl_2 \cdot 4H_2O$, 0.045; $ZnSO_4 \cdot 7H_2O$, 0.0555; $CuSO_4 \cdot 5H_2O$, 0.0195; $CoSO_4 \cdot 7H_2O$, 0.0105; $Na_2MO_4 \cdot 2H_2O$, 0.00675. The ingredients were mixed in 1 liter distilled water. After a month of growth of *P. boryanum*, the culture was used for preparation of the following samples for intraperitoneal (i.p.) injection at the rate (@) of 1 ml per 100 g body weight (b.w.) for each set of 2 male and 2 female mice against parallel controls:

Preparation of Samples: *Treated Series*

T_1=Culture of *P. boryanum*. Pure culture was inoculated to sterile Chu-10 modified growth medium and after a month of culture the sample was ready for use which contained ap-

proximately 3.8 mg (by weight) filaments per 1 ml culture.

T_2=Culture filtrate. 10 ml of 1 month culture was centrifuged at 2000 rpm for 10 min. the supernatant was removed and was recentrifuged as above to make the culture filtrate sample absolutely free from any live *P. boryanum* before use.

T_3=Isolated filaments in phosphate (PO_4) buffer. 10 ml sample of T_1 was centrifuged at 2000 rpm for 10 min. The supernatant was removed and the same volume of PO_4 buffer was added to the tube containing the precipitate (filaments). The suspension was recentrifuged, the supernatant was removed and replaced by equal volume of PO_4 buffer. The process was repeated to remove any trace of Chu-10 medium. The isolated filaments in PO_4 buffer like T_1, were approx. 3.8 mg per ml.

T_4=Heat-killed PO_4 *P. boryanum* suspension. 10 ml of sample T_3 in a tube was put into a water bath containing boiling water for 30 min. which killed all live *P. boryanum* in PO_4 buffer. It was used after verification.

T_5=Cell homogenate. 10 ml of T_3 sample was homogenized with glass bits and the crushed filaments in PO_4 buffer were gently decanted after sedimentation. Then the decanted sample was centrifuged at 10,000 rpm in a refrigerated centrifuge for 15 min. The supernatant containing cell homogenate of *P. boryanum* was injected into mice.

Control Series

C_1=Sterile Chu-10 medium: Before inoculation of *P. boryanum*, the required amount of medium was injected into mice to serve as control of T_1 and T_2.

C_2=PO_4 buffer. Normal mice were injected parallely with same dose of sterile PO_4 buffer to serve as control to T_3, T_4 and T_5 series.

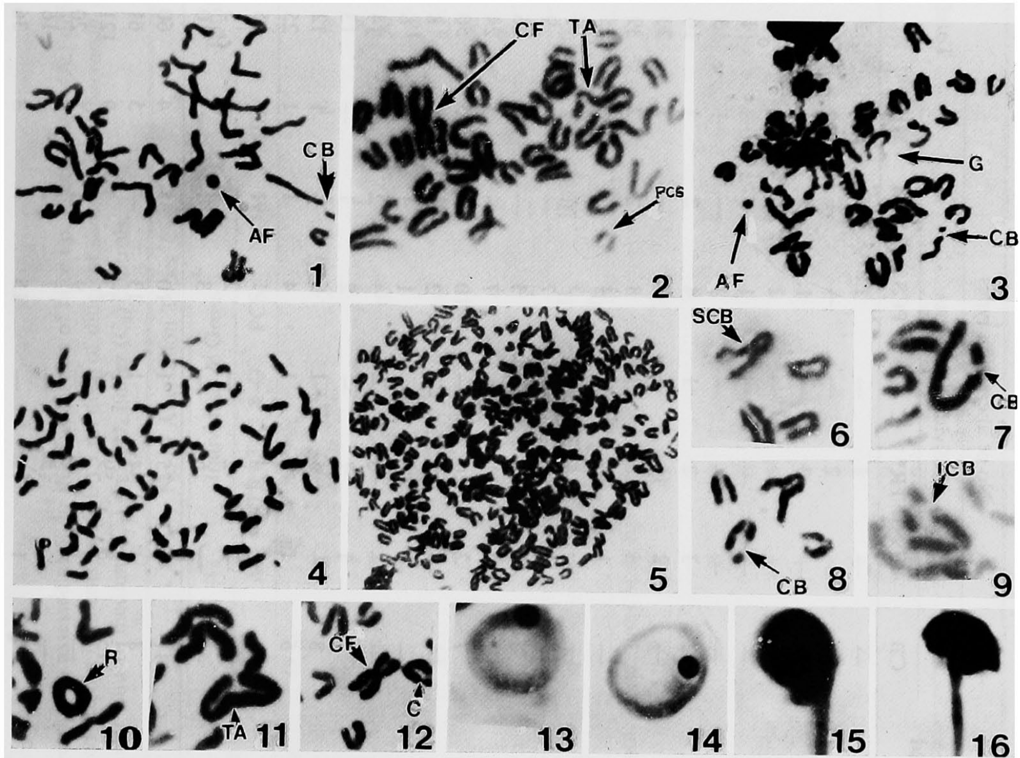
Preparations for Cytogenetic Assays: Following Manna (1985), methods employed were (a) colchicine-sodium citrate-acetic alcohol-flame drying-Giemsa schedule for assessment of bone marrow chromosome aberrations, (b) May-Gruenwald Giemsa staining schedule on bone marrow smear for assessment of poly- and normo-chromatic erythrocytes with micronucleus in micronucleus test (MNT) and (c) smear of epididymal sperm suspension on slides stained with Giemsa for assessment of sperm head abnormalities in treated and control mice.

Results

(a) Bone Marrow (BM) Chromosome Aberrations (CA):

Qualitatively the types of CAs found at different intervals and doses of T_1 and 1 ml dose of T_1, T_2, T_3, T_4 and T_5 were more or less of similar nature (Figs. 1–12) which were chromatid break (CB, Figs. 1, 3, 7, 8), rarely a subchromatid break (SCB, Fig. 6), and isochromatid breaks (ICB, Fig. 9), translocations (TR) in the form of centric fusion (CF, Figs. 2,12), ring (R, Fig. 10), terminal fusion/association (TA, Figs. 2, 11); constriction (C, Fig. 12), gap (G, Fig. 3), and precocious centromeric separation (PCS, Fig. 2) as individual types where some chromosomes within the full complement were affected as against gross types where the entire complement was affected in the form of C-mitosis (Fig. 4), polyploidy (Fig. 5) as numerical change (NUM, Table 1) and pycnosis, stickiness etc. as miscellaneous ones (MIS, Table 1). The frequency and types of CAs in BM were always higher in *P. boryanum* treated specimens than those of control ones but there was no significant difference in effect between two sexes. Out of the total 800 metaphases of 4 intervals in 8 males and 8 females of T_1, the number of CAs was 60 in males and 61 in females and correspondingly in C_1, it was 20 and 16 respectively (Table 1). Combining the data of two sexes, the frequency of CAs was strikingly higher in the treated specimens than that of controls and in the combined data of 4 intervals (1 hr, 24 hr,

48 hr and 1 wk) the difference was highly singnificant ($P<0.001$). The net increase over control was 1.00% at 1 hr, 7.50% at 24 hr, 9.00% at 48 hr, 3.75% at 1 week and 5.30% in the average (Table 1). Further, out of the total 121 CAs in treated specimens, 87 or 71.9% were of undisputed genetic significance comprising breaks, acentric fragments, translocations and polyploids, indicating the genotoxic potential of the culture of *P. boryanum* if other types of aberrations were disregarded. The Chu-10 medium possibly contained some ingredient which was weakly genotoxic as the average CA frequency was 2.3% while in normal mice it was 0.7% comprising mainly physiological types. The frequency distribution of CAs at different in-



Figs. 1–16. Full metaphase complements (Figs. 1–5) and partial metaphases (Figs. 6–12) in bone marrow cells of mice treated with various samples of *P. boryanum*, showing different types of chromosome aberrations as Fig. 1. a chromatid breaek (CB) and an acentric fragment (AF), Fig. 2. a centric fusion (CF), a terminal association (TA) and a precocious centromeric separation (PCS), Fig. 3. a gap (G), an acentric fragment (AF) and a chromatid break (CB) in a sticky metaphase, Fig. 4. a C-mitotic metaphase, Fig. 5. a polyploid metaphase, Fig. 6. a possible subchromatid break (SCB), Fig. 7. a chromatid break (CB), Fig. 8. a chromatid break (CB), Fig. 9. an isochromatid break (ICB), Fig. 10. a ring (R), Fig. 11. terminal associations of three chromosomes (TA), and Fig. 12. a centric fusion (CF) and a constriction (C). Figs 13 and 14, micronucleated poly- and normo-chromatic erythrocytes, respectively. Figs. 15 and 16, sperm with abnormal head morphology.

tervals indicated the effect in T_1 was time-dependednt with a peak at 48 hr and the effect was encountered as early as 1 hr which continued even on 1 week, by the time the cell could have several mitotic cycles (Dewey and Humphrey 1962).

The treatment of 1/2, 1, 2 and 3 ml doses of T_1 showed an appearently dose-dependent increase in CA frequencies though it was not directly proportional to the increase of T_1 doses (Table 2A). The test of correlation yielded a value of $r=0.93$ indicating a positive correlation between dose and effect. Thus the T_1 showed both time- and dose-dependent effects.

The treatment of 1 ml dose of different samples of *P. boryanum* to different sets of mice

Table 1. Bone marrow chromosome aberrations at four intervals in mice separately injected intraperitoneally at the rate of 1 m*l* (contained 3.8 mg filaments per ml) per 100 g body-weight with the culture of *Plectonema broyanum* (T_1) and Chu-10 medium (C_1)

Sample	No. & Sex	Fixation time	Metaphases		No of aberration types									
					Individual & Gross							Total	%	Net % increase
			Total	Affected	CB	AF	TR*	C & G	PCS	NUM	MIS			
T_1	2M	1 hr	200	4	—	—	1	—	1	2	1	5		
do	2F	do	200	2	—	—	2	—	—	—	—	2		
do	Comb	do	400	6	—	—	3	—	1	2	1	7	1.75	1.00
C_1	2M	do	200	2	—	—	—	—	2	—	—	2		
do	2F	do	200	1	—	—	—	—	1	—	—	1		
do	Comb	do	400	3	—	—	—	—	3	—	—	3	0.75	
T_1	2M	24 hr	200	14	3	3	2	1	4	2	1	16		
do	2F	do	200	17	—	2	16	—	4	—	1	23		
do	Comb	do	400	31	3	5	18	1	8	2	2	39	9.75	7.50
C_1	2M	do	200	3	—	1	—	—	4	—	—	5		
do	2F	do	200	4	—	—	1	—	3	—	—	4		
do	Comb	do	400	7	—	1	1	—	7	—	—	9	2.25	
T_1	2M	48 hr	200	22	6	—	15	—	3	—	2	26		
do	2F	do	200	18	1	2	11	—	7	—	3	24		
do	Comb	do	400	40	7	2	26	—	10	—	5	50	12.50	9.00
C_1	2M	do	200	5	—	—	4	—	2	1	1	8		
do	2F	do	200	4	—	—	2	—	1	1	2	6		
do	Comb	do	400	9	—	—	6	—	3	2	3	14	3.50	
T_1	2M	1 wk	200	11	2	1	7	—	2	1	—	13		
do	2F	do	200	9	3	—	5	1	2	—	1	12		
do	Comb	do	400	20	5	1	12	1	4	1	1	25	6.25	3.75
C_1	2M	do	200	5	—	—	4	—	1	—	—	5		
do	2F	do	200	3	—	—	2	—	2	1	—	5		
do	Comb	do	400	8	—	—	6	—	3	1	—	10	2.50	
T_1	8M	Total	800	51	11	4	25	1	10	5	4	60		
do	8F	do	800	46	4	4	34	1	13	—	5	61		
do	Comb	do	1600	97	15	8	59	2	23	5	9	121	7.55	5.30
C_1	8M	do	800	15	—	1	8	—	9	1	1	20		
do	8F	do	800	12	—	—	5	—	7	2	2	16		
do	Comb	do	1600	27	—	1	13	—	16	3	3	36	2.25	

* TR (Translocation) comprised TA/CF/R, for other abbreviations see text.

Table 2. Bone marrow chromosome aberrations assessed at 48 hr in different sets of mice i.p. injected with (A.) different doses of 1 ml culture of *P. boryanum* (contained 3.8 mg filaments) and (B.) 1 ml dose of different samples of *P. boryanum* @ 100 gm b.w. against Chu-10 medium (C_1) and PO_4 buffer (C_2) controls.

Sample	Dose	No of indiv.	Metaphases		No of Aberration Types							Total	%	Net % increase
					Individual & Gross									
			Total	Affected	CB	AF	TR*	C & G	PCS	NUM	MIS			
(A.) Dose-dependent effects of chromosome aberrations at 48 hr														
T_1	1/2 ml	2M+2F	400	31	3	3	26	—	2	1	1	36	9.00	7.00
C_1	do	do	400	7	—	1	2	—	4	1	—	8	2.00	
T_1	1 ml	do	400	40	7	2	26	—	10	—	5	50	12.50	9.00
C_1	do	do	400	9	—	—	6	—	3	2	3	14	3.50	
T_1	2 ml	do	400	45	13	1	25	—	10	4	—	53	13.20	10.70
C_1	do	do	400	9	—	—	4	—	5	1	—	10	2.50	
T_1	3 ml	do	400	56	14	3	29	1	9	5	2	63	15.75	14.50
C_1	do	do	400	4	—	1	2	—	2	—	—	5	1.25	
T_1	Comb	8M+8F	1600	172	37	9	106	1	31	10	8	202	12.62	10.31
C_1	do	do	1600	29	—	2	14	—	14	4	3	37	2.31	
(B.) Different sample-dependent effects of chromosome aberrations at 48 hr														
T_1	1 ml	2M+2F	400	40	7	2	26	—	10	—	5	50	12.50	9.00
C_1	do	do	400	9	—	—	6	—	3	2	3	14	3.50	
T_2	do	do	400	16	2	2	12	—	3	—	1	20	5.00	1.50
C_1	do	do	400	9	—	—	6	—	3	2	3	14	3.50	
T_3	do	do	400	19	3	1	10	—	5	1	1	21	5.25	3.25
C_2	do	do	400	7	—	1	4	—	2	1	—	8	2.00	
T_4	do	do	400	11	—	—	4	—	3	2	2	11	2.75	0.75
C_2	do	do	400	7	—	1	4	—	2	1	—	8	2.00	
T_5	do	do	400	23	2	3	9	—	8	3	3	28	7.00	5.00
C_2	do	do	400	7	—	1	4	—	2	1	—	8	2.00	

* TR=TA/CF/R.

Table 3. Time-dependent effects assessed by (A.) micronucleus test and (B.) sperm head abnormalities is in mice i.p. injected with 1 ml dose (3.8 mg filaments) of *P. boryanum* culture (T_1) against Chu-10 medium (C_1) @ 1 ml per 100 g body weight

	(A.) Micronucleus test								(B.) Sperm head abnormality				
Sample	No & Sex	Time after injection	Erythrocytes					Net % increase	No & Sex	Sperm			Net % increase
			Total	MNP	MNN	Combined	%			Total	Abn. head	%	
T_1	2M+2F	24 hr	8000	23	8	31	0.39	0.31	2M	4000	63	1.57	1.32
C_1	do	do	8000	6	1	7	0.08		do	4000	10	0.25	
T_1	do	48 hr	8000	33	9	42	0.52	0.45	2M	4000	88	2.20	1.68
C_1	do	do	8000	4	2	6	0.07		2M	4000	21	0.52	
T_1	do	1 wk	8000	19	6	25	0.31	0.25	2M	4000	54	1.35	0.97
C_1	do	do	8000	4	1	5	0.06		2M	4000	15	0.38	
T_1	6M+6F	Total	24000	75	23	98	0.40	0.33	6M	12000	205	1.70	1.32
C_1	do	do	24000	14	4	18	0.07		6M	12000	46	0.38	

Abbreviations used: MNP=Micronucleated polychromatic; MNN=Micronucleated normochromatic erythrocyte.

yielded always a higher frequency of CAs of variable degrees than the corresponding controls. The net increase was 9.00% in T_1, 1.50% in T_2, 3.25% in T_3, 0.75% in T_4 and 5.00% in T_5 while the total CA frequencies in treated series only were 12.50% in T_1, 5.00% in T_2, 5.25% in T_3, 2.75% in T_4 and 7.00% in T_5 indicating the maximum genotoxic potential of T_1, followed decreasingly by T_5, T_3, T_2 while it was very weak in T_4 (Table 2B) possibly due to the heating effect.

(b) Micronucleus Test (MNT):

With a view to confirming the genotoxic potential of the culture of *P. boryanum* (T_1) by more than one test as recommended (Bochkov *et al.* 1976), the MNT was conducted in BM cells at 24 hr, 48 hr and 1 week after injecting only once 1 ml dose of T_1 against parallel control mice. Qualitatively both poly (P)- and normo-chromatic (N) erythrocytes (E) contained one micronucleus (MN) when present at different intervals in both treated and control mice (Figs. 13, 14). It was found that the frequency of MN P and N E separately and jointly (MNE) was strikingly higher in T_1 than in C_1 in each interval (Table 3A). In the combined data, the frequency of MNE was higher than control by 0.31% at 24 hr, 0.45% at 48 hr and 0.25% at 1 week. The average increase was 0.33% and the difference was highly significant ($P<0.001$). Among the three intervals, the frequency was highest at 48 hr, showing the same trend as in the case of CA frequencies in BM cells (Table 1).

(c) Sperm Head Abnormalities (SHA):

The Giemsa stained smear of sperm of epididymes of T_1 and C_1 male mice contained various types of SHA (Figs. 15, 16). Out of 4000 sperm assessed in 2 males each of T_1 and C_1 mice (Table 3B), the frequency of SHA was always higher in T_1 than in C_1 and the difference was highly significant ($P<0.001$). The net increase was 1.32% at 24 hr, 1.68% at 48 hr, 0.97% at 1 weeks and 1.32% in the average. Thus SHA data also showed the same trend as in the case of CA and MN test in BM.

Discussion

The mutagenic potential of the blue-green bacterium, *P. boryanum* was found to be positive in all the tests of CA, MN formation and SHA showing the same trend at different intervals commonly assessed. If the data of the genotoxic effects induced by the treatment of the eukaryotic unicellular alga, *Euglena* sp. to experimental mice (Manna and Mohanty 1991) were compared with those of the present prokaryotic bacterium, *P. boryanum*, qualitatively the types of CA, MNE and SHA were of similar nature; even at 24 hr some data of the net increase in frequencies showed closeness because it was 7.25% against 7.50% for CA and 1.62% against 1.32% for SHA in *Euglena* sp. and *P. boryanum* respectively. It might be pointed out that qualitatively the types of CA and MNE in BM cells and SHA in epididymes in mice treated with several species of bacteria, fungi and protozoans were also of similar nature but the frequencies differed (Manna 1992, 1993).

The genotoxic active principle in *P. boryanum* and also in other microbes causing cytogenetic effects remained an unresolved problem. Am attempt was made here to finding out what was associated with the blue-green bacterium, *P. boryanum*. The data of CA for the treatment of 1 ml dose of different samples of *P. boryanum* revealed that the genotoxic agents present in them were of different strengths in decreasing order of T_1, T_5, T_3, T_2 and T_4. The lowest frequency in T_4 might suggest that the agents were heat labile. Anyhow, the active genotoxic principle seemed to be largely associated with the filaments of *P. boryanum* because the net increase in frequency of CA in T_5 (cell homogenate) was 5.00% which was higher than that of undisrupted T_3 (3.25%). Thus the disruption of cell elements of *P. boryanum* by homogenization contained in homogenate principally DNA of filaments. Similarly, one set

of mice treated with DNA-cell-wall fraction of another bacterium, *Xanthobacter flavus* yielded 13.0% CA while another set injected with cytoplasmic fraction yielded 1.3% CA in BM cells (Manna and Sadhukhan, G. C. 1993), supporting that the genotoxic active principle was largely associated with the DNA fraction as found in *P. boryanum*. Further, mice treated separately with sonicated nuclear and non-nuclear fractions of spores of the fungus, *Fusarium oxysporum* yielded strikingly higher frequency of CA in the former than the latter treatment (Manna and Sadhukhan, A. 1990). In bacterium, *Mycobacterium tuberculosis*, the pure protein derivative (P.P.D) is accepted as active principle of toxicity but it was found very weak in producing genotoxic effect in the treated mice as compared to those of log culture, filtrate, isolated bacteria in saline etc. (Manna and Pal 1990a, b). Further studies are in progress to resolve the enigmatic problem of genotoxic active principle associated with *P. boryanum* and other microbial mutagens (Manna 1989).

Summary

The mutagenic potential of the prokaryotic blue-green bacterium, *Plectonema boryanum* in mice treated with its culture against parallel control assayed by chromosome aberration and micronucleus test in bone marrow cells and sperm head abnormalities in epididymes yielded positive results showing time-dependent effect in all the tests and the dose-dependent effect assayed for chromosome aberration only. Further, the treatment of 1 ml dose each of culture (T_1), culture filtrate (T_2), isolated filaments in phosphate buffer (T_3), heat-killed sample (T_4) and cell homogenate-filament DNA (T_5) of *P. borayanum* to different sets of mice against controls yielded net increase in chromosome aberration frequencies at 48 hr of 9.00% in T_1, 5.00% in T_5 3.25% in T_3, 1.50% in T_2 and 0.75% in T_4 in decreasing order, suggesting that the genotoxic active principle was largely associated with filament and it was heat labile. The results of positive genotoxic potential of *P. boryanum* in experimental mice filled up the lacuna of 'Microbes as Living Mutagens' advocated by Manna.

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