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On the Optimization and Application of Sister Chromatid Exchanges (SCEs) and Cytokinesis Blocked Micronuclei (CBMN) in Human Lymphocytes for Cytogenetic Monitoring

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Abstract

The sister chromatid exchanges (SCEs) and micronuclei (MN) are two independent cytogenetic end points widely employed in the study of spontaneous and induced genetic damage in biological systems. A modified simple FPG method and hot salt treatment technique with the considerably reduced time of about 45 and 10 minutes respectively for routine SCE analysis giving excellent reproducibility and clarity of differential staining is reported. The base line SCE frequencies, cell cycle kinetics data from newborns and adult subjects analysed by the hot salt treatment (HST) and fluorescence plus giemsa (FPG) are presented. Identification of late replicating X chromosome and analysis of DNA replication pattern with the application of the modified FPG method in a case study of constitutional X chromosome abnormality (45,X/46,XX/46X,t(X;X)(p;q) observed earlier has also been discussed. The data on baseline MN frequencies by cytokinesis blocked micronucleus assay (CBMN) among different groups of human subjects obtained with different protocols have been summarised. In the final analysis, 6 µg/ml cytochalasin B addition at 24 h following mitogenic stimulation, resulting in a higher percentage of binucleate cells and making scoring faster, in our experience render the CBMN assay, as a more convenient and rapid alternative cytogenetic method for detection of cytogenetic damage in laboratory models or human populations. Proliferation rate index (PRI) in SCE assays and Nuclear division index (NDI) in CBMN assays were found to be better parameters for measuring cell cycle delay than percentage of second division metaphases or binucleate cells alone.

Introduction

Various cytogenetic end points and gene mutations as indicators of DNA damage in somatic and germ cells, form the basis of genotoxicity studies in laboratory models and genetic monitoring of human populations. In fact, cytogenetic

changes such as chromosome aberrations, micronuclei, and sister chromatid exchanges continue to provide the most reliable biological indicators of genotoxic exposure to physical and chemical mutagens in mammalian systems. Unlike *in vitro* test systems using peripheral blood lymphocytes as the target cell, human biomonitoring is essentially an *in vivo - in vitro* test system. SCEs and MN are two independent genetic end points, extensively used in the study of spontaneous and induced genetic damage in various biological systems due to their relative simplicity and rapidity of analysis. SCEs, occurring during chromosome replication are much easier to score than chromosome aberrations, and have proved to be very useful indicators of DNA damage for determining exposure to S-dependent chemicals (Wolff, 1991).

Micronuclei mainly originating from lagging chromosome fragments or whole chromosomes excluded from the main nuclei are indicators of chromosome breakage or spindle dysfunction. While, MN provide a rapid measure of prime chromosome damage, like chromosome breakage or whole chromosome loss, they cannot detect subtle changes such as translocations, inversions etc. The application of cytochalasin B to block cytokinesis, following nuclear division enabled micronuclei to be scored in the intact cytoplasm of binucleate cells that had completed only one nuclear division (Fenech and Morley, 1985, 1986). The scoring of MN in these cytokinesis blocked binucleate cells (CBMN) enhanced the reliability and sensitivity of the method to a great deal. Further, the application of antikinetochore antibodies or *in situ* hybridization with centromeric DNA probes, chromosome specific or chromosome region specific probes can distinguish between micronuclei induced by the two mechanisms and yield information regarding the chromosomal origin of micronuclei (Heddle, 1991). A modified simple FPG method and hot salt treatment technique, which considerably reduced the time required for obtaining good sister chromatid differentiation (SCD), for routine SCE analysis is presented here. Likewise, a modified CBMN procedure as a more convenient method for cytogenetic monitoring in human lymphocytes is summarised. Both, cytochalasin B concentration and the time of its addition to the cultures are important variables that influence the baseline MN frequencies, as well as the percentage of binucleate cells. The present experimental protocol consisted of the analysis of baseline MN frequencies following 6 µg/ml cytochalasin B addition at 24 or 44 h after mitogenic stimulation. The data on baseline frequency of SCEs and MN in human lymphocytes of different population groups scanned during the course of studies undertaken, with the methods optimised in our laboratory are summarised in this communication. Identification of late replicating X chromosome and analysis of DNA replication pattern with the application of the modified FPG method in a case study of constitutional X chromosome abnormality (45,X/46,XX/46X,t(X;X)(p;q) observed earlier (Krishnaja, *et al.*, 1993) has also been discussed.

Materials and methods

Heparinised blood samples were obtained from adult healthy volunteers. Within 24 h of collection of samples whole blood cultures were initiated by a standard procedure used in our laboratory as reported elsewhere (Krishnaja and Sharma, 1991). For each culture, 4 ml Hams F10 medium with 200 mM. L-glutamine, 0.5 ml foetal bovine serum, 0.1 ml reconstituted PHA were inoculated with 0.3 ml whole blood. No antibiotics were added to the cultures at any stage. BrdU was added at a final concentration of 10 µg/ml at the begining of the cultures. The cultures were terminated after 72 h incubation at 37°C, following a final 3 h treatment with colcemid at a concentration of 0.02 µg/ml and harvested following the conventional procedure involving hypotonic KCl treatment, fixation with methanol - acetic acid (3:1) and air drying on chilled wet slides.

A modification of the FPG method (Morimoto *et al.*, 1982) was applied to obtain harlequin chromosomes. Chromosome preparations aged two days were stained in Hoechst 33258 (100 µg/ml in distilled water) for 20 min. After rinsing in tap water slides were mounted in Sorensen's buffer (M/15, pH 8.0 adjusted with 5% NaOH) under a coverslip and exposed to 360 nm light from a Black ray lamp (distance 2cm, 20 $J/m^2/sec.$) for 12 min on a slide warming tray at 60°C. Finally, slides rinsed in ice cold Sorensen's buffer pH 6.8 followed by tap water were stained in 4% Giemsa (Merck) in Sorensen's buffer pH 6.8. Optimum staining time varied between slide and stain batches, but was of the order of about 8 min. For the identification of the late replicating X-chromosome, BrdU at a concentration 200 µg/ml was added to the whole blood cultures, 7 h prior to harvest at 72 h. Slides were then processed using the FPG method exactly in the same manner as mentioned above (Sharma and Krishnaja, 1996).

The hot salt treatment for SCD was essentially the same as reported by Sakanishi and Takayama (1977). In this case instead of foetal bovine serum, 20% heat inactivated human AB serum was used in the culture medium. The slides were incubated in $Na_2 HPO_4$, 1.0 M, pH 8.8 at 75°C for 2 min; rinsed briefly in distilled water, stained in 4% Giemsa (Merck) in Sorensen's buffer, pH 6.8 for about 5-7 min (Krishnaja and Sharma, 1991).

The procedure employed for CBMN assay was essentially the same as reported earlier (Krishnaja and Sharma, 1994). The present experimental design consisted of 6 µg/ml cytochalasin B, added at 24 or 44 h., after initiation of cultures to induce binucleate cells. The cultures were terminated at 72 h. A mild hypotonic treatment with 0.8% cold KCl for 5 min. preceded the fixation with standard methanol-acetic acid 3:1 fixative for 10 min. The second addition of fixative contained 1% formaldehyde, that enhances the preservation of cytoplasm. After one more addition of fixative, cells were gently dropped on wet chilled slides and stained with Merck's Giemsa stain 1% in phosphate buffer pH 6.8 for 20 min.

A total of 50 second division metaphases were analysed for SCE from each subject and the results have been presented as frequency of SCE per cell. Further, about 200 metaphases were classified according to the staining pattern to determine the number of M1, M2, M3 and M4 ≥ division metaphases. The proliferation rate index (PRI) was calculated according to Lamberti *et al.*, 1983. In case of MN, 1000 binucleated CB cells with well preserved cytoplasm were scored per donor. The criteria for identification of MN and CB binucleate cells was that of Countryman, 1976, Heddle *et al.*, 1990; Krishnaja and Sharma 1994. A minimum of 1000 cells were scored to evaluate proportion of bi, tri and tetranucleated (multinucleated) cells. Nuclear division index was calculated as NDI = [M1 + (2 x M2) + (3 x M3) + (4 x M4)/N], where M1-M4 represent the number of cells with 1 to 4 nuclei, respectively. N is the total number of cells scored. (Eastmond and Tucker, 1989).

Results and discussion

The FPG method and the hot salt treatment (HST) procedure presented above are preferred for routine analysis of SCEs, because of their excellent reproducibility, distinct chromosome morphology and clarity of differential staining (see Fig. 1a, b, c, d, e, f). Further, the considerably reduced time of about 10 and 45 minutes, in case of HST and FPG, respectively for achieving such quality preparations is another important additional feature. The frequency of SCEs, cell cycle kinetics and proliferation rate index of 10 adults analysed by the FPG method are given in Table 1. The mean baseline SCE frequency based on FPG method in 10 healthy adults was found to be 6.15 ± 0.34 SCE/cell with range from 4.76 to 7.72. The PRI ranged from 1.75 and 2.25 among the various subjects. Cell cycle analysis showed 11-42% first, 43-73% second, 4-32% third and 0-9% fourth division metaphases. The base level of SCEs, cell cycle kinetics data from newborns and adults analysed by the two different methods outlined above have been presented in Table 2. The mean SCE frequency observed in cord blood samples in our laboratory among 10 newborns by the hot salt treatment technique for SCD was 5.56 ± 0.21 SCE/cell and in 5 adults 7.50 ± 0.21 SCE/cell (Krishnaja and Sharma, 1991). No statistically significant gender dependent difference was found in the frequency of SCEs among the newborns. This is commensurate with the abundant evidence of lack of sex dependence of SCEs in literature. Whenever an increased frequency of SCEs is reported in females, it has been explained by the difference in the level of hormones (Das, 1988). A statistically significant elevation was found, when adult blood samples (non-smokers, difference 1.94 SCE/cell p, 't test) were compared with cord blood samples (Krishnaja and Sharma, 1991). In the adult samples, the mean SCE value was 7.50 2.1 SCE/cell. Cell cycle analysis in cord blood samples revealed 17-22% first, 60-80% second, 3-20% third and 0-2% fourth division metaphases, compared to adult blood samples, which revealed 17-38% first, 56-68% second, 2-15% third and 0-1% fourth division metaphases, respectively.

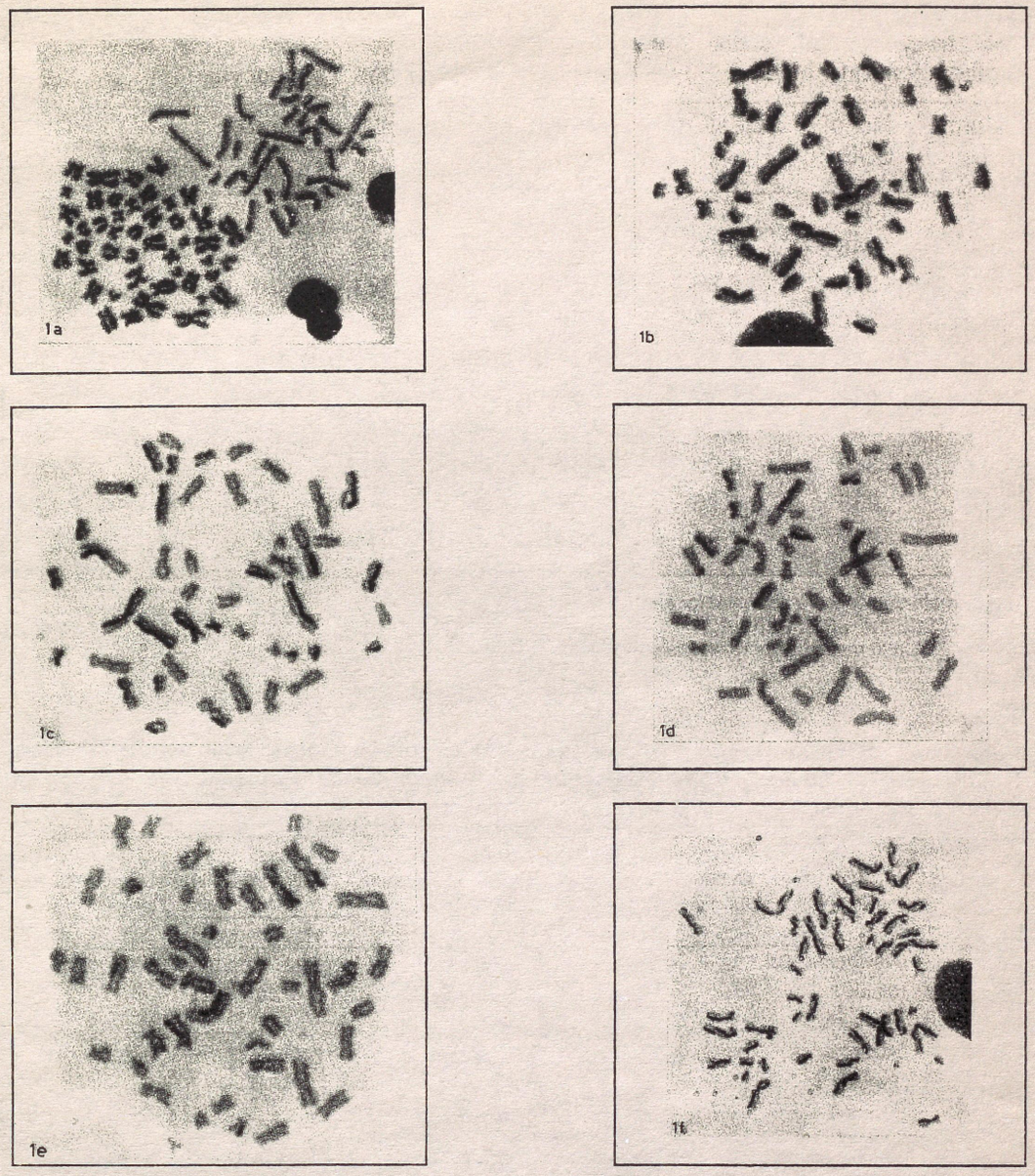


Fig. 1: Sister chromatid differentiation based on BrdU incorporation in human lymphocyte chromosomes demonstrated by FPG staining. (a) First and second, (b) second (c) third (d, e) fourth ≥ cell cycle metaphase spreads, (f) SCEs induced by Ascorbic acid plus Mitomycin C treatment.

Table 1
The frequency of sister chromatid exchanges (SCEs), cell cycle kinetics and proliferation rate index (PRI) in human peripheral blood lymphocytes

Donor No.	No. of SCEs*	SCEs/Cell**	Range	Cell cycle kinetics				PRI***
				M1	M2	M3	M4	
1	386	7.72	2-18	45	106	31	18	2.11
2	245	4.90	1-11	28	124	43	5	2.13
3	298	5.96	2-15	53	121	26	0	1.87
4	312	6.24	2-14	34	130	32	4	2.03
5	323	6.46	3-13	57	125	17	1	1.81
6	245	4.90	1-11	26	114	42	18	2.26
7	302	6.04	2-11	44	146	8	2	1.84
8	346	6.92	4-13	84	86	26	4	1.75
9	238	4.76	2-11	30	121	43	6	2.13
10	382	7.64	3-14	22	110	64	4	2.25

* Fifty metaphases were analysed for each donor
** Mean SCE/cell ± S.E. 6.15 ± 0.34
*** For PRI, 200 metaphases were analysed for each donor.

Table 2
Base level of SCEs in cord blood lymphocytes of newborns and peripheral blood lymphocytes of adults. Combined data from normal human subjects assayed by two different methods.

Method	No. of Subjects	Culture duration	No. of meta-phases scored	Mean SCE/cells ± SE	Range	Cell cycle kinetics percentage of cells (Range)			
						M1	M2	M3	M4
Hot salt	Newborns 10	72 h	500	5.56 ± 0.21	2-11	17-22	60-80	3-20	0-2
treatment	Males 5		250	5.59 ± 0.36	2-11				
technique	Females 5		250	5.53 ± 0.21	2-11				
	Adults 5	72 h	250	7.50 ± 0.21	2-17	17-38	56-68	2-15	0-1
	Female 1								
	Males 4 (26-58 Y)								
FPG	Adults 10	72 h	500	6.15 ± 0.34	1-18	11-42	43-73	4-32	0-9
	Males (31-54 Y)								

The base level frequencies of SCEs among (5.08/cell to 24.00.cell) normal healthy subjects reported from different laboratories vary significantly (Das 1988). Several factors including biological, genetic, personal habits, life style and cultural practices have been implicated in the variation in baseline SCE frequencies (Carrano and Natarajan, 1985). The short lived nature of the SCE lesion is

often considered as a limiting factor for population surveillance studies during acute mutagenic exposure, as it persists only for few days to weeks after the exposure. However, due to its high sensitivity, relative procedural simplicity and inherent ability to produce readily quantifiable data, SCE assays continue to be a method of choice for detection of genotoxicity in human lymphocytes and laboratory mammals. The exact mechanism and biological significance of SCE formation, however remain to be elucidated (Tucker *et al.*, 1993) The extent of sister chromatid differentiation, degree of resolution and the frequency of SCEs has been reported to vary markedly due to methodological consideration (Morgan and Crossen, 1981). The reproducibility and quality of preparations achieved by the two methods reported here can augment the routine analysis of SCEs for laboratory investigations and population monitoring during occupational or environmental exposures.

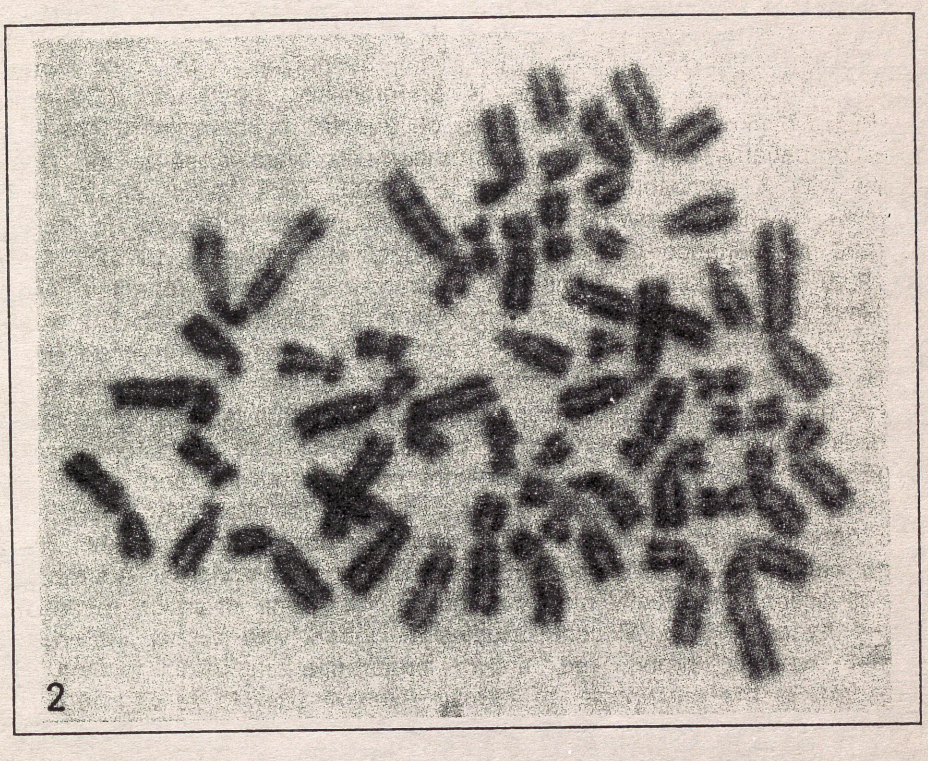


Fig. 2 : FPG stained human lymphocyte metaphase spread showing late replicating translocation (X) chromosome - lightly stained.

In a case study of constitutional X chromosome abnormality (6%) 46, XX (32%) 45,X/(62%) 46,X,t (X; X)(p: q), the FPG method was applied to identify the late replicating X chromosome (Fig.2). A terminal pulse BrdU administered in whole blood cultures, selectively suppressed Hoechst fluorescence in late replicating regions, which had incorporated BrdU. Replication studies in 50

metaphases demonstrated that the translocation X was always preferentially inactivated with reference to the normal X chromosome. The normal X chromosome was found to be early replicating in all the cells analysed. Analysis of the replication characteristics of the translocation X chromosome showed a terminal replication pattern highlighting bands q25 and q27 even in the translocated regions. Few cells displayed a predominant pattern in which bands p21 and q21 were also accentuated. The findings of normal karyotypes in the parents of the proband revealed this to be a de novo case of translocation, as has been observed in most published cases. Nearly all reported cases of Xq anomalies have been found to be sporadic and not familial. It is also well known that the Xq defects are usually not transmitted, because they lead to reduced fertility of women and affect viability of the male fetus, due to lack of an intact X chromosome. In the proband, mixoploidy with 45 X and normal 46, XX cell lines suggested a post-zygotic origin in the early stages of development. Monosomy for even very small segments of the X chromosome and trisomy for other segments may negatively influence the final phenotype. In most females, a structurally abrnomal X chromosome is preferentially inactivated (Therman and Patau, 1974), minimising the occurrence of genetic imbalances. The resultant phenotype of patients with structural rearrangements were close to the phenotype of patients with X monosomy (Luleci *et al.,* 1990; Tuck Muller *et al.,* 1993). In the seventies, a model based on DNA methylation was proposed to explain the initiation and maintenance of mammalian X-inactivation (Riggs, 1975). Giacalone *et al.,* (1992) had reported a new X chromosome specific repetitive sequence, a 3 kb Hind III clone with a base composition of 63% C+G, which was found to be highly methylated in active X chromosome, and hypomethylated in the inactive X chromosome. Studies with antibodies against the acetylated form of Histone H4 on metaphase chromosomes, had shown that although the inactive X can be distinguished from the active X by its overall lack of histone acetylation, three regions of hyperacetylation are present on both human and mouse inactive X chromosomes (Jeppessen and Turner, 1993). Initiation of X-inactivation is known to depend on the presence of a unique region or locus known as the X inactivation centre or XIC defined by the study of chromosomal rearrangements. Its mode of action is currently unknown, but is the scene of much research effort (Heard and Avner, 1994). Recent studies in normal and Turner individuals with X-centromere specific probe, has shown preferential inclusion of the inactive X-chromosome in the micronuclei of human females (Hando *et al.,* 1997).

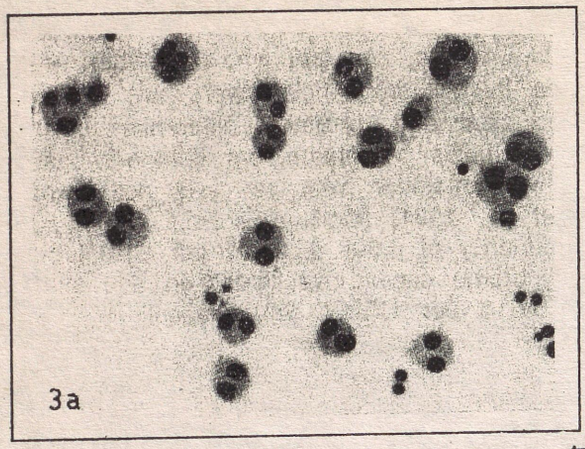
The data on baseline micronuclei frequencies in the different groups studied with different protocols have been summarised in Table 3 and Figs. 3a, b and c. The distribution of mono, bi, tri, tetra (and above) nuclei, the nuclear division index and baseline MN frequencies in ten healthy donors with 6 µg/ml Cyt.B in cultures added at 24 h are, presented in Table 4. The mean micronuclei per CBBN cell frequency in 14 subjects (23-58 y) was found to be 0.011 ± 0.001 and 0.10 ± 0.001 per CBBN cell when 6 µg, Cyt.B was added to the cultures at 24 or 44 h, respectively. The mean micronuclei frequency in 15 normal subjects and 15 thalassaemia traits was found to be 0.0125 0.001 and 0.0231

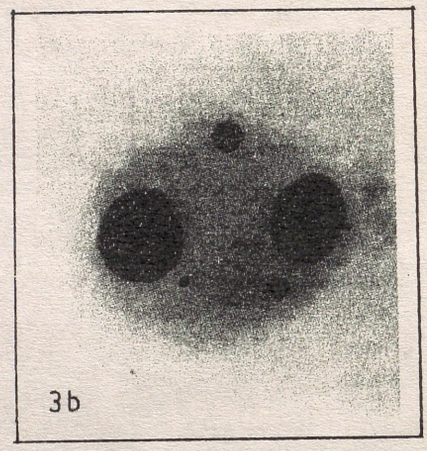
0.0026 per CBBN/cell, respectively at 3 µg Cyt.B/ml at 44h. While, analysing the thalassaemia traits (TTs) data, factors like age, sex and smoking habits were looked into, but sex related differences in MN frequencies were not noticed. Fenech and Morley (1994) had indicated clear and consistent differences in the spontaneous MN frequency of males and females. Although, our data are based on limited number of subjects, a positive correlation with sex has not always been found in MN assays (Tomanin *et al.*, 1991, Migiliore *et al.*, 1991). In paediatric referral group, a lower MN frequency of 0.006 ± 0.001 per CBBN cell, was observed. In the three Downs syndrome cases investigated in this study, MN frequencies of 0.001, 0.001 and 0.016 per CBBN cell, respectively were noticed.

Table 3
Baseline micronuclei frequencies in binucleated cytokinesis blocked (CB) peripheral blood lymphocytes from human subjects

Gps.	No. of subjects class details		Cyt.B concentration µg/ml	Time of Cyt. B addition (h)	Micronuclei/ cell ± SE	Range	Cells with ≥ 3 MN
I	Normal	15	3.0	44	0.0125±0.001	5-19	1
	Males	14					
	Female (27-54 Y)	1					
II	β-thalassaemia		3.0	44	0.0231 ± 0.0026	8-40	4
	traits	15					
	Males	8					
	Females (11-40 Y)	7					
III	Normal	14	6.0	44	0.010 ± 0.001	2-18	0
	Females	2					
	Males (29-54 Y)	12					
IV	Normal	14	6.0	24	0.011 ± 0.01	3-19	3
	Females	2					
	Males (23-58 Y)	12					
V	Paediatric		6.0	44	0.006 ± 0.001	1-16	0
	Referrals	5					
	Females	2					
	Males (11 days-12 Y)	3					
VI	Downs		6.0	44	0.006 ± 0.001	1-16	0
	syndrome	3					
	Female	1					
	Males (11 days-3 months)	2					

One thousand CBBN cells scored per donor.
Standard errors are based on the Poisson distribution.
Thal. Trait SE based on the heterogeneity of the population. - Krishnaja and Sharma (1994).





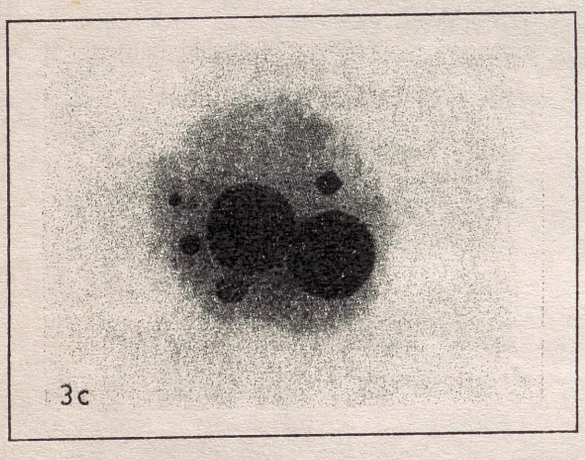


Fig. 3: Cytochalasin-B blocked human lymphocytes showing micronucleus. (a) A typical low power field of cells. (b) and (c); Binucleate CB cells with 3 and 4 MN each in the cytoplasm.

Table 4
Distribution of cells with different number of nuclei and baseline micronuclei frequencies in CBBN cells, after 6 μg/ml Cyt.B addition at 24 h. in whole blood cultures from 10 healthy donors.

Donor	No. of cells with indicated numbers of nuclei				Nuclear Division index-NDI	Micronuclei/CB BN cells
	1	2	3	4 or above		
1.	354	537	72	37	1.79	0.007 ± 0.002
2.	326	546	87	41	1.84	0.008 ± 0.002
3.	343	591	35	31	1.75	0.012 ± 0.003
4.	315	595	62	28	1.80	0.004 ± 0.002
5.	240	590	80	90	2.02	0.010 ± 0.003
6.	212	537	130	121	2.16	0.009 ± 0.003
7.	325	563	75	37	1.82	0.003 ± 0.001
8.	172	535	139	154	2.27	0.011 ± 0.003
9.	241	500	125	134	2.15	0.019 ± 0.004
10.	323	576	68	33	1.81	0.005 ± 0.002

NDI - one thousand cells scored per donor.
MN - one thousand CBBN cells scored per donor.

In achieving optimum cytokinesis arrest of human lymphocytes, the concentration of cytochalasin B and time of addition are important variables. The optimum concentration and time of addition of Cyt.B for induction of BN lymphocytes, reported in literature ranged from 3 to 6 μg/ml between 16 to 68 h of culture incubation (Fenech and Morley, 1985; Lee *et al.,* 1994; Ford *et al.,* 1988; Koksal *et al.,* 1989). Cyt.B was even added at the initiation of lymphocyte cultures, in a pioneer study by Riddler and Smith (1968). Addition of 6 μg/ml Cyt.B at 24 h following mitogenic stimulation did not result in a higher MN yield in the present study. In fact the mean MN frequencies in binucleated cells was lower at 6 μg/ml compared to 3 μg/ml Cyt.B in a collaborative study carried out to ascertain the reproducibility of CBMN assay in two laboratories (Surralles *et al.,* 1992). Significant dose related effects of Cyt.B on the frequency of binucleate cells with MN have not been observed (Ellard and Parry, 1993, Migliore *et al.,* 1994). The percentage of binucleated cells and NDI obtained by 6 μg/ml Cyt.B added at 24 h of culture ranged between 50.0 to 59.5 and 1.8 to 2.3, respectively in 10 healthy donors (Table 4). Slightly lower MN frequencies, higher values for NDI and percentage of binucleated cells were encountered when Cyt.B was added at 24 h. NDI was found to be a better parameter for measuring cell cycle delay than percentage BN cells, as can be depicted from Table 4.

The spontaneous MN frequency obtained in normal subjects in this study was of the same order as observed by other authors. The baseline MN frequencies in human lymphocytes has been reported to show a wide variation ranging from 2 to 67 per 1000 BN cells (Huber *et al.,* 1992). The wide variation seen in MN frequencies is a reflection of the degree of pre-existing *in vivo* chromosome damage (Fenech and Morley, 1997) and may be related to various

endogenous and exogenous factors, as discussed earlier in this communication for SCEs and by others elsewhere (Migliore *et al.,1993). Evaluation of inter-individual variations in the base level MN frequencies are important in the estimation of in vivo* low dose radiation or mutagenic exposure, based on MN calibration curves (Almassy *et al.,* 1987, Huber *et al.,* 1992). Size distribution analysis and use of antikinectochore antibodies have indicated that the baseline MN frequency may be mainly a reflection of mitotic dysfunction (Wakata and Sasaki, 1987; Fenech, 1993). In the final analysis, 6 µg/ml Cyt.B added at 24 h, following mitogenic stimulation resulting in a higher percentage of binucleate cells and making scoring faster in our experience, render the CBMN assay as a more convenient and rapid alternative cytogenetic method for detection of chromosomal damage in laboratory models or human populations.

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