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Preferential Sensitivity of Acrocentric Chromosomes to the Aneugenic Effect of Colchicine†

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In order to evaluate the predisposition to the aneuploidy-inducing agent colchicine (Col) on lymphocytes from trisomic 21 patients compared with their parents and with a control group of subjects without trisomic children, we performed the micronucleus (MN) assay associated with C-banding, CREST staining, and nucleolar organizing region (NOR)-banding. According to our results Col behaves as an aneugenic agent independently of the population studied for CREST and C-banding. The Col-induced MN exhibited a clear majority (>80%) of positive NOR-MN, meaning that they contain a NOR region transcriptionally active or inactive. The same data were observed in trisomic 21 individuals, their parents, and the control group, without significant differences between them. These results seem to suggest a preferential effect of the aneugen Col on acrocentric chromosomes in all of the three groups studied.

Key words: Down syndrome, acrocentric chromosome, colchicine, micronuclei, C-banding, CREST staining, N-banding

INTRODUCTION

Chromosomal abnormalities are believed to contribute significantly to human reproductive failure, carcinogenesis, and other pathophysiological conditions [1]. In addition, chromosomal abnormalities are believed to play an important and possibly essential role in neoplastic development [2–4]. Based on the evidence for the involvement of structural and numerical aberrations in these pathophysiological conditions, rapid techniques for the identification of aneuploidy-inducing and clastogenic

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†Dedicated to Professor Luis Archer on the occasion of his 70th birthday.

agents in mammalian cells have been developed. One approach that has been successfully employed involves the use of the micronucleus (MN) assay in cytokinesis-blocked lymphocytes [5,6] combined with several techniques allowing the analysis of the nature of DNA content of the MN, such as centromeric heterochromatin by C-banding [7], kinetochore by antikinetochore antibody isolated from scleroderma patients (CREST antibody) [8,9], and fluorescent in situ hybridization using either centromeric or specific probes [10].

Trisomy 21 is a major cause of mental retardation combined with congenital heart disease, congenital anomalies of the gastrointestinal tract, defects of the immune and endocrine systems, an increased risk of leukemia, and an Alzheimer-like dementia [11–13]; moreover, it is the prototype for the study of human aneuploidy. Non-disjunction was very soon recognized as the cause of the malsegregation leading to free trisomy accounting for 95% of Down syndrome (DS) patients [13]. In fact, an increased mitotic non-disjunction in parents of trisomic children and in couples with recurrent abortions has been demonstrated [14]. The data obtained support the idea that errors of chromosome segregation may be due to spindle defects, to chromosome breakage, or to abnormal chromosome associations [14].

In this work we performed the MN assay associated with C-banding or CREST staining to evaluate the predisposition to the aneuploidy-inducing agent colchicine (Col) on lymphocytes from trisomic 21 patients compared with their parents and with a control group of subjects without trisomic children. In addition, we analyzed the predisposition to the induced aneuploidy of acrocentric chromosomes in the same groups of donors. Considering that acrocentric chromosomes contain defined nucleolar organizing regions (NOR) in the short arm, carrying the ribosomal RNA genes [15], we performed the NOR-banding technique associated with the MN test, by using the NaH_2PO_4 plus Giemsa method that allows the identification of transcriptionally active or inactive NORs [16]. NOR-banding has been used before in metaphase cells, namely to assess preferential non-disjunction of acrocentrics [17,18].

MATERIALS AND METHODS

Samples

Three groups of subjects were studied. They included 1) 8 couples with a trisomic 21 child; 2) 9 DS patients with free trisomy (7 males and 2 females); and 3) 8 individuals karyotypically normal without trisomic children or recurrent abortions (control group).

Lymphocyte Cultures

Cultures were set up by adding 0.5 ml of whole blood to 4 ml of Ham's F-10 medium (Sigma, St. Louis, MO) supplemented with 24% fetal calf serum (FCS; Sigma), antibiotics (penicillin 50 UI/ml and streptomycin 50 μg/ml; Sigma), 1% of heparin (Braun Melsungen AG), and 1% L-glutamine (Sigma). Lymphocytes were stimulated by using 20 μl of phytohemagglutinin (PHA; HA 15, Murex, Dartford, England) and incubated at 37°C.

Chemical Treatment

Twenty-four hours after initiation, cultures were treated with Col at final concentrations of 0, 25, 50, 100, and 150 nM during 19 hr for the MN assay.

Cytokinesis-Blocked Method

Cytochalasin-B (Cyt-B; Sigma) stock solution was prepared in dimethylsulfoxide (DMSO) at a final concentration of 4 mg/ml and stored at –20°C. The stock solution was thawed and added to 44 hr cultures at a final concentration of 12.5 μM. Cultures were harvested 28 hr after Cyt-B addition.

Harvesting and Slide Preparation

At 72 hr of incubation, the cultures were harvested by centrifugation at 319*g* for 10 min, washed twice with RPMI 1640 medium (Flow, McLean, VA) supplemented with 2% FCS, and the pellet was resuspended in hypotonic solution (RPMI 1640-distilled water, 1:4 + 2% FCS). Smears were fixed in acetic acid-methanol (1:3 v/v) for C- and N-banding or fixed in absolute methanol precooled to –20°C during 30 min for CREST assay.

C-Banding

The method used was essentially that described in the literature [7,19] with some optimization steps to interphase cells. Slides aged 1–3 weeks were immersed in 0.2 N HCl for 20 min at room temperature and rinsed in distillate water, then transferred to 5% aqueous $Ba(OH)_2$ at room temperature for 40 min, and subsequently rinsed in distilled water. After this treatment the slides were incubated in 2× SSC at 60°C for 90 min and rinsed in distilled water and finally stained in 4% Giemsa (pH 6.8 in phosphate buffer) for 4–20 min.

CREST Staining

The method used was essentially as described [8] with some modifications. Briefly, the kinetochores were tagged with the antikinetochore antibody serum (Antibodies, Inc., Davies, CA) diluted 1:1 in phosphate buffered saline (PBS) with 0.1% Tween-20 during 30 min in a humidified chamber at 37°C. Unbound antibodies were removed by five washing steps of 5 min each with PBS-1% bovine serum albumin (BSA; Sigma). Fluorescein isothiocyanate (FITC)-conjugated goat secondary anti-human IgG antibody (Amersham, Buckinghamshire, United Kingdom) was diluted 1:16 in PBS-BSA and coupled to the first antibody by a 45 min incubation in the same conditions. Unbound secondary antibodies were removed as described previously, plus five washing steps of 2 min each with PBS pH 7.2. In order to reduce fading of fluorescence the preparations were mounted in a mixture of antifade solution (Vectashield, Vector, Burlingame, CA) with propidium iodide (4 μg/ml). These preparations were scanned using a Nikon epifluorescent microscope fitted with filter B (515 nm range). Immunofluorescence studies were carried out only on cells where both nuclei exhibited several yellow spots and cytoplasm was preserved.

N-Banding

The technique used was essentially as described [16,19] with some adaptations to interphase cells. The slides were immersed in 1 M Na_2HPO_4 (pH 4.2) at 96°C for 8–15 min, rinsed in distilled water, and stained with 4% Giemsa (pH 6.8, phosphate buffer) for 10–20 min.

Slide Scoring

For each dose a total of 1,000 cytokinesis-blocked (CB) cells with preserved cytoplasm were scored. Scoring of MN was made using only the slides on which the

treatment clearly allows visualization of stained MN. Only CB cells with preserved cytoplasm and with both nuclei marked were scored. Slides were analyzed with a Leitz microscope at magnifications of ×1,000. For the fluorescent slides a Nikon epifluorescent microscope fitted with filter B (515 nm range) was used.

Statistical Analysis

The chi-squared test was used for testing the significance of dose-response data. Comparisons between the three populations and the C-banding, CREST, and N-banding were performed by using the t-test for independent samples.

Cytotoxicity Analysis

As a measure for cytotoxicity or cell cycle delay, we used the mitotic index (I_M) [20], given by

$$\frac{(\text{CB} + 2 \times \text{Poly N})/\text{N from treated samples}}{(\text{CB} + 2 \times \text{Poly N})/\text{N from untreated samples}} \times 100$$

where CB = the cytokinesis-blocked lymphocytes, Poly N = the polynucleated lymphocytes, and N = the total number of cells scored. Values of I_M below 50% were considered an indicator of cytotoxicity or cell cycle delay.

RESULTS

The dose-response data for the Col-induced MN in the three groups studied are shown in Tables I–III, respectively, for control group, parents of trisomic 21 children, and DS subjects.

According to our results, Col induced a significant dose-dependent increase in the MN frequency for all of the donors studied. No significant differences (t-test for independent samples) were found between the three groups of subjects.

The highest dose of Col was shown to be cytotoxic in all groups of donors exhibiting a I_M <50%. Trisomic 21 individuals and their parents appeared to be more sensitive to the genotoxicity of Col since the second highest dose is also cytotoxic (I_M <50%). On the basis of these results we chose 50 nM as the optimum dose for the remainder of the experiments. This choice represents a compromise between a high induced MN frequency and no cytotoxicity or cell cycle delay.

The results on the nature of the DNA content of induced MN for the three groups of donors are shown in Table IV. Figure 1 shows examples of MN showing the presence of centromeric DNA (C^+-MN) after C-banding or CREST staining (K^+-MN), or the presence of NOR regions after N-banding (N^+-MN).

Our C-banding (Fig. 2) and CREST immunoassay data (Fig. 3) reveal that a majority (>80%) of the induced MN contain centromeres (MN-C^+) or kinetochores (MN-K^+), indicating their origin from whole chromosomes. These data are common to the three groups of donors studied and no significant differences (t-test for independent samples) were found between the frequencies of MN-C^+ and MN-K^+ among them. After C-banding or CREST staining, no MN with more than one spot were found.

N-banding was applied (Fig. 4) to estimate the sensitivity of acrocentric chromosomes to the aneugenic effect of Col on the three groups studied. Our results show that Col induces a majority of MN-N^+ (>80%) for all donors, indicating the

TABLE I. Frequencies of MN Induced by Col on Lymphocytes From the Control Group[†]

Donor	Dose (nM)	% CB	% Poly N	% MNCB	% Mono MN	% Met	I_M
C1	0	53.1	10.6	5	0.2	0.7	
	25	49.8	7.8	38*	0.2	0.3	88.0
	50	39.6	5.9	50*	0.2	0.1	69.2
	100	30.1	4.0	67*	0	0.1	51.3
	150	21.2	2.1	83*	0	0	34.2
C2	0	43.7	5.2	6	0	0.6	
	25	41.9	4.9	28*	0	0.3	95.6
	50	39.2	4.1	43*	0	0.1	87.6
	100	37.2	3.1	64*	0	0	80.2
	150	21.3	1.8	81*	0	0	46.3
C3	0	52.3	4.3	5	0.8	0.3	
	25	48.1	3.9	24*	0.3	0.1	91.8
	50	47.2	3.5	33*	0.1	0.1	89.0
	100	31.3	2.4	41*	0	0	59.3
	150	20.4	1.0	74*	0	0	36.8
C4	0	48.9	5.2	6	0.6	0.1	
	25	42.1	4.1	27*	0.2	0.1	84.8
	50	37.1	3.4	39*	0	0	74.3
	100	33.1	2.8	46*	0	0	65.3
	150	20.3	2.0	72*	0	0	41.0

[†]CB = cytokinesis-blocked cells; Poly N = polynucleated cells; MNCB = CB cells with MN; Mono MN = lymphocyte with MN; Met = metaphases; I_M = mitotic index.
*$P < 0.001$: significance according to chi-squared test.

TABLE II. Frequencies of MN Induced by Col on Euploid Lymphocytes From Parent of Trisomic 21 Children[†]

Donor	Dose (nM)	% CN	% Poly N	% MNCB	% Mono MN	% Met	I_M
P1	0	50.2	5.7	7	0.2	0	
	25	39.9	3.8	37*	0	0.1	77.1
	50	33.6	3.0	49*	0	0.1	64.3
	100	21.3	2.5	67*	0	0	42.7
	150	19.0	1.8	82*	0	0	36.7
P2	0	44.8	4.2	8	0	0.3	
	25	40.1	3.1	34*	0	0.1	87.0
	50	36.0	2.6	52*	0	0.2	77.4
	100	22.0	1.1	73*	0	0	45.5
	150	17.3	0.5	85*	0	0	34.4
P3	0	53.2	6.6	5	0	0.2	
	25	49.7	5.4	21	0	0	91.1
	50	41.8	4.9	38*	0	0	77.7
	100	37.8	3.3	42*	0	0	66.9
	150	21.4	2.1	57*	0	0	38.6
P4	0	53.3	6.2	2	0	0.1	
	25	49.3	5.7	29*	0	0	92.4
	50	40.6	8.8	43*	0.2	0.1	88.6
	100	35.1	3.6	65*	0	0	64.4
	150	22.0	2.2	87*	0	0	40.2

[†]CB = cytokinesis-blocked cells; Poly N = polynucleated cells; MNCB = CB cells with MN; Mono MN = lymphocyte with MN; Met = metaphases; I_M = mitotic index.
*$P < 0.001$: significance according to chi-squared test.

TABLE III. Frequencies of MN Induced by Col on Lymphocytes From Trisomic 21 Patients[†]

Donor	Dose (nM)	% CB	% Poly N	% MNCB	% Mono MN	% Met	I_M
D1	0	49.7	4.8	9	0.2	0.6	
	25	43.1	4.3	32**	0	0.3	87.2
	50	41.7	3.0	41**	0	0.2	80.4
	100	29.0	2.2	46**	0	0.3	56.3
	150	21.1	1.5	52*	0	0.3	24.1
D2	0	49.1	5.5	8	0.1	0.4	
	25	40.3	4.6	27**	0	0.1	82.4
	50	39.1	3.5	35**	0	0	76.7
	100	29.1	2.4	44**	0	0.1	56.4
	150	24.1	1.7	50**	0	0	45.8
D3	0	48.4	4.7	9	0.1	0.5	
	25	42.1	3.6	24*	0.1	0.2	85.3
	50	39.8	2.3	32**	0	0.1	76.8
	100	23.1	2.0	44**	0	0	46.9
	150	21.3	1.4	51**	0	0	41.7
D4	0	43.6	4.6	10	0	0.4	
	25	39.9	3.5	31**	0	0.2	88.8
	50	33.9	2.9	42**	0	0	75.2
	100	27.1	2.1	49**	0	0	59.3
	150	18.5	1.5	54**	0	0	40.7

[†]CB = cytokinesis-blocked cells; Poly N = polynucleated cells; MNCB = CB cells with MN; Mono MN = lymphocyte with MN; Met = metaphases; I_M = mitotic index.
*$P < 0.01$, **$P < 0.001$: significance according to chi-squared test.

presence of acrocentric chromosomes in the induced MN. No MN with more than one spot were found. No significant differences (t-test for independent samples) were found among the control group, trisomic 21 patients, and their parents.

DISCUSSION

It is known that Col prevents the polymerization of tubulin by forming a Col complex at the microtubular ends leading to chromosome lag with resultant aneuploidy [21]. However, Col, as other mitotic inhibitors, also alters the structure of the

TABLE IV. Average Percentages and Total of C-Banding, CREST, and N-Banding-MN Induced by Col on Control Donors (C), Parents of Trisomic 21 (P), and DS Subjects (D)[†]

Studied group	Dose (nM)	MNCB % C^+	MNCB Σ	MNCB % K^+	MNCB Σ	MNCB % N^+	MNCB Σ
C	0	29.6	8	29.6	8	23.6	7
	50	76.6*	34*	80.3*	35*	86.9*	32*
P	0	22	11	16.4	7	16.4	9
	50	86.9*	41*	86.7	40*	86.7*	38*
D	0	22	11	25.2	11	25.2	10
	50	86.9*	36*	80.4*	36*	80.4*	33*

[†]MNCB = cytokinesis-blocked cells with percentages of positive MN (C^+, K^+, or N^+) and total (Σ) number of micronuclei per 1,000 CB.
*$P < 0.001$: significance according to chi-squared test.

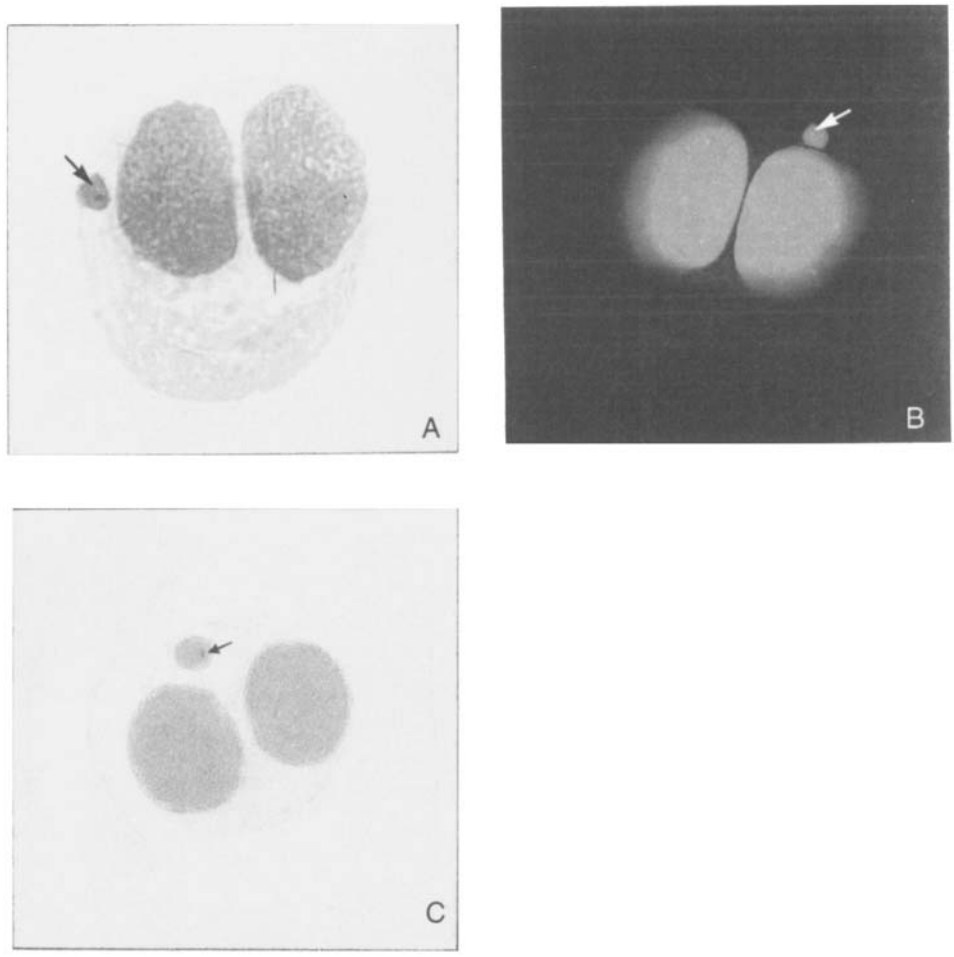

Fig. 1. MN-containing CB lymphocyte induced by Col (**A**) after C-banding [the arrow indicates the centromeric DNA (MN-C^+)]; (**B**) after CREST staining [the arrow indicates the fluorescent kinetochore (MN-K^+)]; and (**C**) after NOR-banding (the arrow indicates the NOR region).

kinetochore over a wide dose range [22]. A MN contains a whole chromosome if it results from chromosome lag identified as a centromeric/kinetochore-positive MN [8,9]. To avoid false negative results due to the damage of kinetochores, we performed our study by using both CREST staining and C-banding.

Our results with CREST and C-banding are in agreement with the well-known aneugenic behavior of Col independently of the population studied. No increase of C^- or K^- was found for any of the donors. Trisomic 21 patients and their parents show similar average percentages of induced MN-C^+ and -K^+ compared to the percentages obtained for the control group. For untreated cultures the majority (>50%) of MN obtained were negative, meaning that they contain acentric fragments. Trisomic 21 subjects exhibit higher percentages of spontaneous positive MN, but the difference is not significant when compared with their parents and the control group.

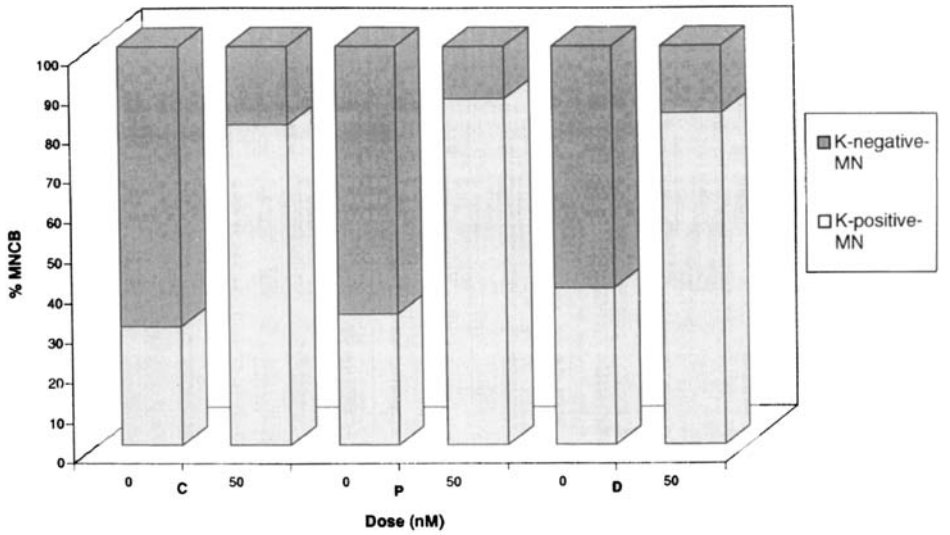

Fig. 2. Percentage of C-banding-induced MN by Col on lymphocytes from the control group (C), parents of trisomic 21 children (P), and DS subjects (D).

Our data with NOR-banding show that the MN background does not contain preferentially NOR regions identified as negative MN. However, the MN induced by Col exhibited a clear majority (>80%) of positive NOR-MN, meaning that they contain a NOR region transcriptionally active or inactive. The same data were observed in trisomic 21, their parents, and the control group, without significant differences between them.

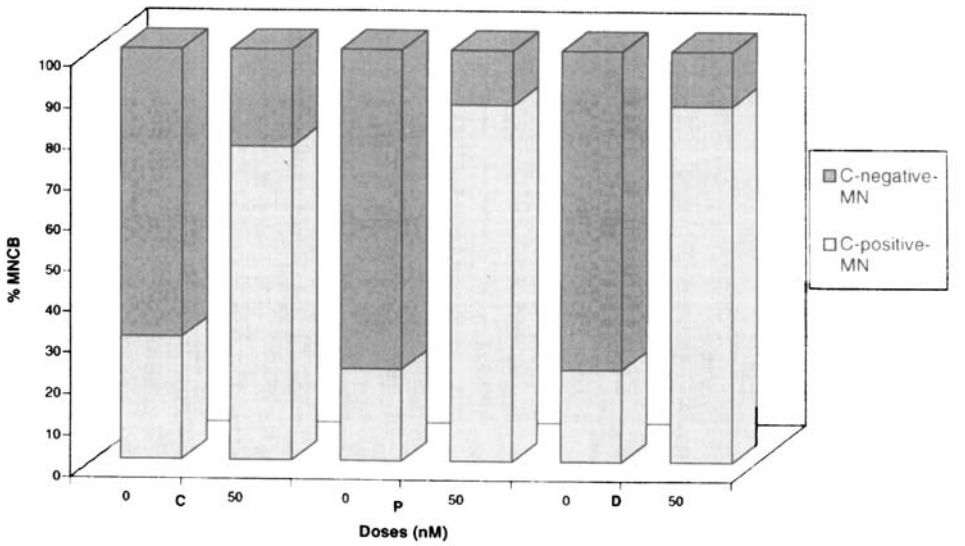

Fig. 3. Percentage of CREST-induced MN by Col on lymphocytes from the control group (C), parents of trisomic 21 children (P), and DS subjects (D).

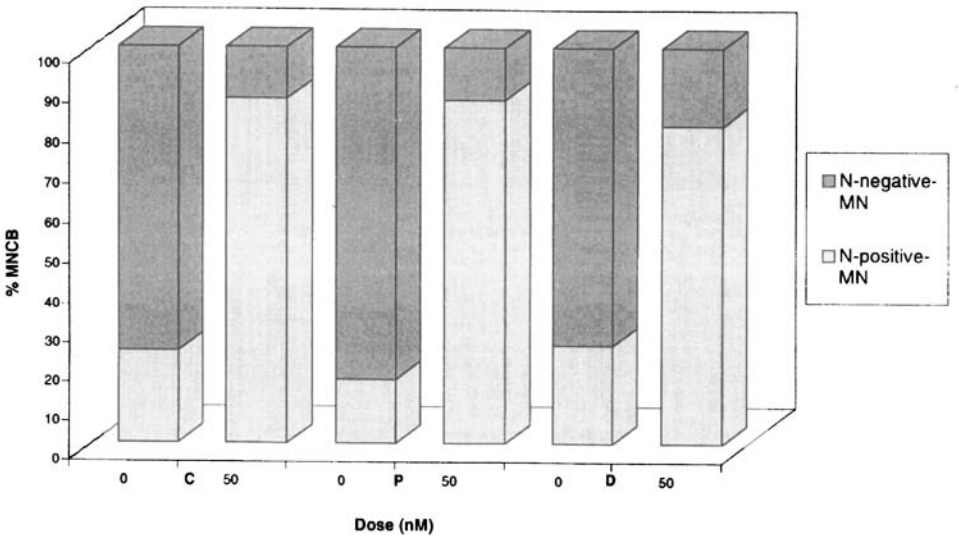

Fig. 4. Percentage of N-banding-induced MN by Col on lymphocytes from the control group (C), parents of trisomic 21 children (P), and DS subjects (D).

It is very interesting to note that the induced percentages of C^+ and K^+-MN are quite similar to the NOR-positive ones. These results seem to suggest a preferential effect of the aneugen Col on acrocentric chromosomes in all of the three groups studied.

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REFERENCES

1. De Braekeleer M, Dao TN: Cytogenetic studies on male infertility: A review. Hum Reprod 6:245–250, 1991.
2. Yunis JJ: The chromosomal basis of neoplasia. Science 221:227–236, 1983.
3. Oshimura M, Barret JC: Chemically induced aneuploidy in mammalian cells: Mechanisms and biological significance in cancer. Environ Mutagen 8:129–159, 1986.
4. Fearon ER, Volgstein BA: Genetic model for colorectal tumorigenesis. Cell 61:759–767, 1990.
5. Fenech M, Morley A: Measurement of micronuclei in human lymphocytes. Mutat Res 147:29–36, 1985.
6. Fenech M, Morley A: Cytokinesis-block micronucleus method in human lymphocytes: Effect of in vivo aging and low dose X-irradiation. Mutat Res 161:193–198, 1986.
7. Versachaeve L, Vanderkerken K, Kirsch-Volders M: C-banding as a simple tool to discriminate between micronuclei induced by clastogens and aneugens. Stain Technol 63:351–354, 1988.
8. Degrassi F, Tanzarela C: Immunofluorescent staining of kinetochores in micronuclei: A new assay for the detection of aneuploidy. Mutat Res 203:339–345, 1988.
9. Eastmond DA, Tucker JD: Identification of aneuploidy inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. Environ Mol Mutagen 13:34–43, 1989.

10. Eastmond DA, Pinkel D: Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence in situ hybridisation with chromosome-specific DNA probes. Mutat Res 234:303–318, 1990.
11. Epstein CJ: The consequences of chromosome imbalance. Am J Med Gen Suppl 7:31–37, 1990.
12. Korenberg JR, Kawashima H, Pulst SM, Allen L, Magenis E, Epstein CJ: Down syndrome: Toward a molecular definition of the phenotype. Am J Med Gen Suppl 7:91–97, 1990.
13. Kola I: Molecular genetics of Down syndrome. Reprod Fertil Dev 1:81–83, 1989.
14. Staessen C, Maes AM, Kirsch-Volders M, Susanne C: Is there a predisposition for meiotic nondisjunction that may be detected by mitotic hyperploidy? Clin Genet 30:31–36, 1983.
15. Henderson AS, Warburton O, Atwood KC: Location of ribosomal DNA in the human chromosome complement. Proc Natl Acad Sci USA 69:3394–3398, 1972.
16. Verma R, Babu A: In: "Human Chromosomes: Manual of Basic Techniques." New York: Pergamon Press, 1989, pp 5–10.
17. Hens L, Kirsch-Volders M, Arrighi F, Susanne C: Relationship between measured chromosome distribution parameters and Ag staining of the nucleolus organizer regions. Hum Genet 53:363–370, 1980.
18. Mattei MG, Mattei JF, Vidal I, Giraud F: Advantages of silver staining in seven rearrangements of acrocentric chromosomes, excluding Robertsian translocations. Hum Genet 54:365–370, 1980.
19. Martin PK, Rowley JD: An improved technique for sequential R-, Q-, and C-banding bone marrow chromosomes. Stain Technol 58(1):7–12, 1983.
20. Van Hummelen P, Elhajouji A, Kirsch-Volders M: Clastogenic and aneugenic effects of three benzimidazole derivatives in the in vitro micronucleus test using human lymphocytes. Mutagenesis 10(1):23–29, 1995.
21. Margolis RL, Wilson L: Addition of colchicine-tubulin complex to microtubule ends: The mechanism of substoichiometric colchicine poisoning. Proc Natl Acad USA 74:3466–3470, 1977.
22. Onfelt A: Mechanistic aspects on chemical induction of spindle disturbances and abnormal chromosome numbers. Mutat Res 168:249–300, 1986.