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Micronuclei in cytokinesis-blocked lymphocytes as an index of occupational exposure to antineoplastic drugs

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In order to investigate possible DNA damaging effects of antineoplastic drugs, the micronucleus assay combined with Giemsa, DAPI and silver staining was performed. Blood samples were taken from nurses working without adequate protection in oncological department on preparing and administration of different antineoplastic drugs. Lymphocytes were cultivated in vitro at 37°C. To prevent cytokinesis, at 44h cytochalasine-B at a final concentration of 3μg/ml was added. The results obtained indicate statistically significant increases in the total number of micronuclei in the exposed subjects compared to controls. DAPI staining has revealed signal-positive and signal-negative micronuclei while silver staining has revealed Ag-NOR$^+$ and Ag-NOR$^-$ micronuclei. Compared to controls, the number of signal-positive and Ag-NOR+ micronuclei in the exposed subjects were increased, indicating a greater susceptibility of particular chromosomes to damage caused by antineoplastic agents.

Key words: antineoplastic agents-adverse effects; occupational exposure; lymphocytes; micronucleus test

Introduction

Antineoplastic drugs are inhibiting or preventing growth of neoplasms, by checking the maturation and proliferation of malignant cells. They are risk factors for different categories of workers who are occupationally exposed during the stocking, preparation, administration and disposal of such agents.[1-4] Many of these, commonly used in cancer chemotherapy have proved carcinogenic, mutagenic and teratogenic in experimental animals and *in vitro* test systems.[5-9] Careless handling of cytotoxic agents may lead to exposure of the personnel to amounts detectable by chemical or biological methods in the body fluids or cell samples of the subjects. The exposure is typically to mixed compounds over a longer period, and to low exposure levels with accidental peaks. Therefore, the use of biological exposure markers is appropriate for monitoring such exposure patterns. The biological markers/methods for exposure assessment are either non-specific (e.g. cytogenetic damage, point mutations or ^{32}P-post-labelling adducts in peripheral blood lymphocytes, urinary mutagenicity) or specific for a given compound (immunological methods, specific analytical methods).[3,10-15]

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The *in vitro* micronucleus assay is used widely as a useful endpoint in biomonitoring, and ecotoxicology, as well as for the assessment of *in vitro* and *in vivo* damage of chromosomal material caused by different mutagens.[16-21]

The aim of this study was to investigate the cytogenetic damage resulting from the exposure to different antineoplastic drugs in nurses working in oncology department under inadequate protection. Genotoxic damage in lymphocytes was evaluated by a micronucleus assay. For sensitive detection of genome damage conventional Giemsa staining was compared to DAPI and silver staining techniques.

Materials and methods

Subjects

Samples of peripheral blood were taken from 20 healthy never-smoking subjects aged from 24 to 50 years (mean age 39.5 years). Ten of them were controls and ten were nurses working in oncological department of one hospital. Nurses were daily involved in the preparation and administration of different antineoplastic drugs for an average period of 17.3 years. Both experimental groups were previously interviewed to document a history of radiation exposure, chemical exposure and viral infection within one month before the study.

Micronucleus assay

Blood samples were cultured at 37°C *in vitro* in F-10 medium (Gibco) supplemented with fetal bovine serum (Biological Industries, Israel), phytohaemagglutinin (Murex) and antibiotics (penicillin and streptomycin). Cultures were harvested at 72 h.

To prevent cytokinesis, at 44 h Cytochalasin-B (Sigma) in the final concentration of 3 μg/ml was added to each sample, and the cells were harvested after a further incubation of 28 h. The slides for scoring micronuclei were prepared according to the modified method of Fenech and Morley.[22] After a brief treatment with physiological saline, cells were fixed with 3:1 mixture of methanol and acetic acid. They were dropped onto clean slides, dried at room temperature and afterwards stained using conventional Giemsa staining, DAPI and silver staining technique.

Staining techniques

Giemsa staining was performed by means of 5% buffered solution of Giemsa for 10 minutes. After staining, the slides were washed and air-dried.

DAPI (4',6-diamidino-2-phenylindol-dihydrochloride) staining was performed according to Schweizer.[23] Prior the staining, slides were preincubated in Mc Ilvaine's buffer (citric acid - disodium hydrogenphosphate) pH 7.0 for 10 minutes. The staining solution contained 1 μg of DAPI / ml of Mc Ilvaine's buffer (pH 7.0). The staining procedure was carried out in dark and lasted for 10 minutes. Afterwards, slides were rinsed in Mc Ilvaine's buffer, air-dried and mounted in 1:1 mixture of glycerol and Mc Ilvaine's buffer. The preparations were observed under short-wave-length blue light using an UG 1 filter for excitation.

For the silver staining of NORs the method of Howell and Black[24], which uses gelatine as colloidal protector, was employed. To prepare colloidal developer, gelatine was dissolved in deionized water by stirring and gentle heating. When the gelatine was dissolved, formic acid was added. To prepare silver nitrate solution, silver nitrate was dissolved in deionized water. To stain NORs, a colloidal developer and $AgNO_3$ solution (50%) were mixed and pipetted onto the micronuclei preparations. The slides were covered with coverslips and

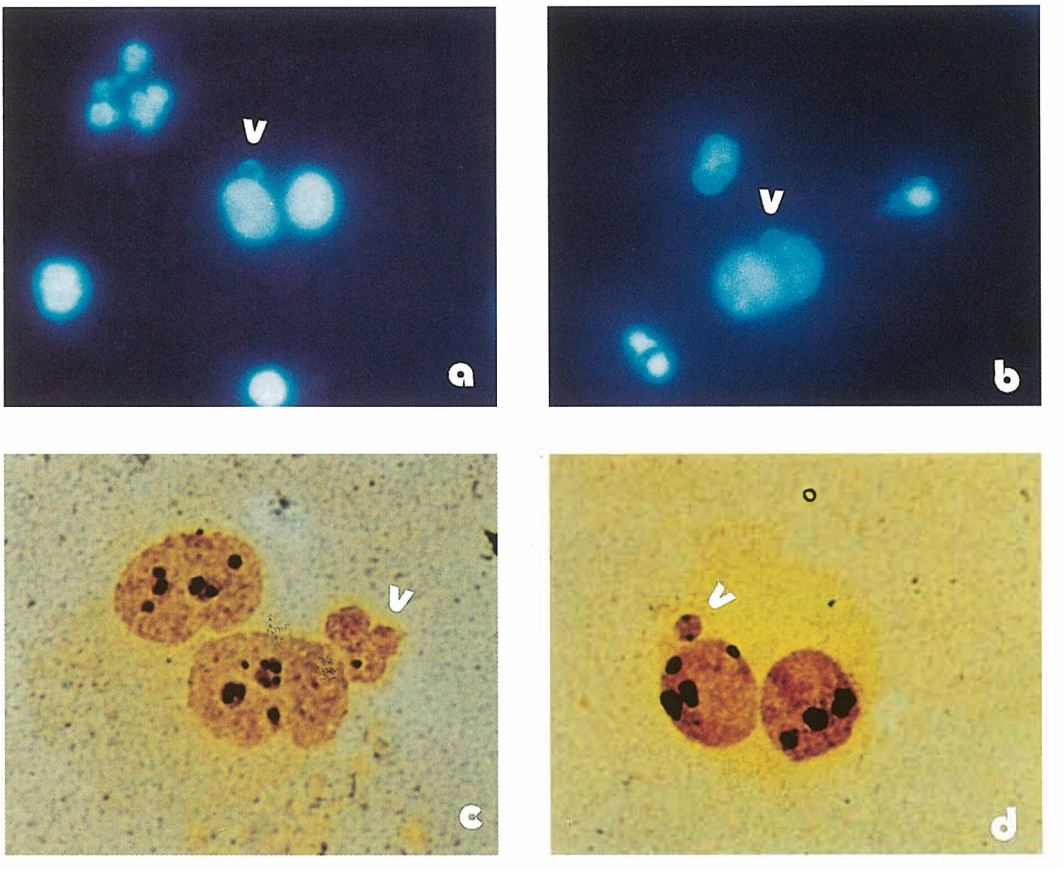

Figure 1. Binucleated lymphocytes of nurses occupationally exposed to antineoplastic drugs. (a) signal-positive micronucleus stained with DAPI, (b) signal-negative micronucleus stained with DAPI, (c) cell with two Ag-NOR$^-$ and one Ag-NOR$^+$ micronuclei after silver staining, (d) Ag-NOR$^+$ micronucleus with two signals after silver staining

placed on a hot plate pre-heated to 70°C. After 1-2 minutes the solution turned yellow and then golden brown. At this stage slides were washed off, rinsed with deionized water and air-dried.

Scoring of the slides

Slides from the exposed and control subjects were scored by the same scorer.

For the incidence of micronucleated lymphocytes and determination of the frequency of micronuclei 500 binucleated cells per subject were scored.

The published criteria for MN determination were followed: (1) binucleated cells containing any number of MN were scored; (2) fluorescence intensity per unit area of scorable MN was either equal in intensity or more or less intense than that of the main nuclei; (3) only MN that were distinctly separate from the main nuclei and located within binucleated cells with intact cytoplasmic and nuclear membranes were scored. Micronuclei were located for their DAPI fluorescence and NOR activity and successively classified as signal-positive and signal-negative as well as Ag-NOR$^+$ and Ag-NOR$^-$ MN.

Table 1. Total number and distribution of micronuclei in lymphocytes of control group after Giemsa, DAPI and silver staining

Subject No.	1	2	3	4	5	6	7	8	9	10
No. of	496	498	497	496	498	498	498	497	497	498
binucleated	496	497	497	497	496	498	498	497	498	497
cells										
without MN	495	497	497	497	496	497	497	498	496	497
No. of	3	2	2	4	4	2	2	3	3	2
binucleated	4	3	3	3	3	2	2	2	2	3
cells										
with 1 MN	5	3	3	3	4	3	3	2	4	3
No. of	1	0	1	0	1	0	0	0	0	0
binucleated	0	0	0	0	1	0	0	1	0	0
cells										
with 2 MN	0	0	0	0	0	0	0	0	0	0
Total No. of	5	2	4	4	6	2	2	3	3	2
MN per 500	4	3	3	3	5	2	2	4	2	3
binucelulated	5	3	3	3	4	3	3	2	4	3
cells										

Giemsa staining technique

DAPI staining technique

Silver staining technique

Statistical analysis

The statistical significance of the results was determined using the χ^2 test.

Results

The results regarding the frequency, distribution and total number of micronuclei (MN) for the control group are reported in Table 1, and for the exposed group in Table 2.

All staining techniques have revealed statistically significant increases in total number of micronuclei in the exposed group ($P<0.05$ using χ^2 test) compared to the controls. The distribution of micronuclei per 500 binucleated cells in all the exposed subjects was also disturbed compared to the controls.

Considering the presence or absence of bright DAPI, flourescent dots inside of MN signal-positive and signal-negative MN have been detected. Their total number and distribution for the exposed and control groups are shown in Table 3. In all the exposed subjects we have observed an increased total number of micronuclei as well as an increased number of signal-positive micronuclei compared to control.

Related to the patterns of nucleolar organizer (NOR) activity, Ag-NOR$^+$ (MN that contain one or more NOR parts) and Ag-NOR$^-$ micronuclei (MN without NOR parts) have been noticed. Their total number and distribution of in control and exposed subjects are shown in Table 3. In all the exposed subjects an increased total number of micronuclei as well as increased number of Ag-NOR$^+$ micronuclei, compared to the controls, is observed.

Figure 1 shows signal-positive and signal-negative micronuclei obtained after staining with DAPI (a,b) and Ag-NOR$^+$ and Ag-NOR$^-$ micronuclei (c,d) obtained after silver staining in subjects occupationally exposed to antineoplastic drugs.

Table 2. Total number and distribution of micronuclei in lymphocytes of exposed group after Giemsa, DAPI and silver staining

Subject No.	1	2	3	4	5	6	7	8	9	10
No. of	477	482	488	490	489	490	488	491	462	485
binucleated	488	478	480	482	485	480	490	483	474	484
cells										
without MN	488	482	483	477	486	486	480	485	487	483
No. of	13	16	12	10	10	8	11	8	32	12
binucleated	11	17	19	13	14	17	11	15	24	15
cells										
with 1 MN	8	14	15	17	14	12	18	12	19	15
No. of	0	1	0	0	1	2	1	0	5	2
binucleated	1	4	1	3	1	2	0	2	1	1
cells										
with 2 MN	4	4	2	5	0	2	1	2	3	5
No. of	0	1	0	0	0	0	0	1	1	0
binucleated	0	0	0	1	0	1	0	0	1	0
cells										
with 3 MN	0	0	0	1	0	0	1	1	0	0
No. of	0	0	0	0	0	0	0	0	0	0
binucleated	0	0	0	2	0	0	0	0	0	0
cells										
with 4 MN	0	0	0	0	0	0	0	0	0	0
Total No. of	13	21	12	10	12	12	13	11	45	18
MN per 500	13	25	21	30	16	24	11	19	29	17
binucelulated	16	22	19	30	14	16	23	19	25	19
cells										

Giemsa staining DAPI staining Silver staining

Discussion

The primary source of human exposure to antineoplastic drugs results from their use in cancer therapy. However, persons involved in the manufacture, preparation and administration of drugs to patients and in nursing care of patients may also be exposed.

The results of numerous studies on cytogenetic endpoints performed on medical personnel exposed to antineoplastic drugs are conflicting, probably due to different degrees of exposure in different occupational settings, as well as due to different sensitivity of the indicators, to their different persistence and different safe handling measures. [10,13,17,25,26]

Based on their mode of action, antineoplastic agents are divided into several categories. Since most of these drugs exert their effects during a certain phase of the cell cycle (cell growth phase, cell division phase, resting phase etc.), many treatment regimens combine two or more of these agents. This is the reason why are nurses usually exposed to a mixture of different antineoplastic drugs used in their daily preparation and administration procedures. The most frequently handled antineoplastic drugs in our study were: bleomycin, vinblastine, cyclophosphamide,

Table 3. Total number and distribution of DAPI signal-positive and signal-negative micronuclei (MN) as well as Ag-NOR$^+$ and Ag-NOR$^-$ MN for control and exposed subjects

Subject No.	1	2	3	4	5	6	7	8	9	10
Exposed group										
No. of signal-positive MN	8	10	13	15	10	14	8	8	7	4
No. of signal-negative MN	5	17	8	12	6	10	3	11	22	13
Total No. of MN	13	27	21	27	16	24	11	19	29	17
No. of Ag-NOR$^+$MN	8	15	15	13	12	10	14	15	15	12
No. of Ag-NOR$^-$MN	8	7	4	17	2	6	9	4	10	7
Total No.of MN	16	22	19	30	14	16	23	19	25	19
Control group										
No. of signal-positive MN	2	1	2	1	2	0	1	1	0	1
No. of signal-negative MN	2	2	1	2	2	2	1	2	2	2
Total No. of MN	4	3	3	3	4	2	2	3	2	3
No. of Ag-NOR$^+$MN	2	2	1	2	2	1	2	1	1	2
No. of Ag-NOR$^-$MN	1	3	2	2	1	2	1	1	3	1

DAPI staining technique Silver staining technique

cisplatinum, 5-fluorouracil, adriamycin and mitomycin C.

The results of our study have clearly indicated that occupationally exposure to antineoplastic drugs caused cytogenetic damage. The in vitro micronucleus assay combined by Giemsa, DAPI and silver staining techniques has revealed a significant increases in the number of micronuclei as well changes in their distribution in all the exposed subjects compared to control.

It is known that micronuclei originate from either whole chromosomes or acentric chromosome fragments due to chromosomal breakage, or from lagging chromosomes which consequently are excluded from the main nuclei. Therefore, enumeration of MN can provide an index of chromosome loss from the main nuclei if whole chromosomes can be identified within them. The incidence of micronuclei observed could result from clastogenic as well as aneugenic effect on peripheral blood lymphocytes. It has been shown that lymphocytes are an extremely sensitive indicator of induced chromosome structural damage both *in vivo* and *in vitro*. Approximately 90% of lymphocytes have a half-life of three years and thus can reflect damage incurred over a long period.

Among the antineoplastic drugs used in our study some are known aneuploidy-inducing agents with spindle damaging effects (vinblastine) while others are clastogens with direct DNA damaging effects (bleomycin, mitomycin C). Considering the average duration of occupational exposure to those agents, an increased number of micronuclei observed in all the exposed subjects, compared to controls, is not surprising. These results are consistent with previous reports on the use of different endpoints with different antineoplastic drugs in vivo and *in vitro*.[8,12,17,27,28,29,30]

In this study, the micronucleus assay was performed in combination with conventional Giemsa staining and more specific DAPI and silver staining. Giemsa technique was compared to DAPI and silver because it is known that both techniques exhibit a considerable specificity in detecting particular chromosomal regions or distinct chromosomes. Therefore, they allow us to speculate about the ori-

gin of micronuclei. It is known that DAPI staining produces intense fluorescence of the paracentromeric regions of chromosomes 1,9,16, of the distal part of the long arm of Y chromosome, and also of a region of the short arm of chromosome 15. On the other hand, silver staining make visible nucleolar organizing regions (NORs), which are loops of chromatin containing rRNA gene clusters. In normal human cells, NORs are localized on the secondary constrictions of the 10 acrocentric chromosomes of D and G groups.

Previous reports have shown that the frequency of micronuclei detected by special staining techniques was generally higher than with conventional May-Grünwald Giemsa staining technique.[29] Our results are consistent with this observation (Tables 1,2).

The incidence of DAPI signal-positive and Ag-NOR$^+$ micronuclei lead us to a conclusion that parts of regions which they detect specifically are involved in acentric fragments, or in whole chromosomes excluded as micronuclei from the main nucleus due to clastogenic or aneugenic effect of antineoplastic drugs mixture.

In the exposed subjects an increased number of both types of micronuclei compared to controls was observed. Thus, it is reasonable to assume that chromosomes of D and G groups, respectively 1,9,16, 15 and Y, are more susceptible to DNA damage caused by antineoplastic drugs, as compared to other human chromosomes.

The results of the present investigation show that inadequate protection in handling antineoplastic drugs leads to a significant cytogenetic damage. Our study confirms the suitability of micronucleus assay, combined with special staining techniques for the assessment of risk of occupational exposure to antineoplastic drugs.

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