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Biological Effect Monitoring in Peripheral Blood Lymphocytes from Subjects Occupationally Exposed to Antineoplastic Drugs: Assessment of Micronuclei Frequency

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Abstract: Biological Effect Monitoring in Peripheral Blood Lymphocytes from Subjects Occupationally Exposed to Antineoplastic Drugs: Assessment of Micronuclei Frequency: Milena VILLARINI, *et al*. Department of Medical-Surgical Specialties and Public Health (Section of Public Health), University of Perugia, Italy—Objectives: Antineoplastic drugs (ANPDs) are widely used in the treatment of cancer and some nonneoplastic diseases. However, most if not all of these chemical agents are generally nonselective and, along with tumor cells, normal cells may undergo cytotoxic/genotoxic damage. Italian pharmacists and nurses occupationally exposed to ANPDs during their normal work routines were monitored to evaluate biological effects (*i.e.*, cytogenetic damage) eventually associated with exposure. The subjects were also monitored for primary, oxidative and excision repaired DNA damage as evaluated by comet assay (published data). In the present paper, we present the results obtained with the cytokinesis-block micronucleus (CBMN) test. **Methods:** The CBMN test in peripheral blood lymphocytes was applied because of its ability to detect both clastogenic and aneugenic effects, and because it has recently been reported that micronuclei (MNs) are predictive of cancer risk in human populations. In this study, the evaluation of MN frequency was carried out using the CBMN test in the absence or in the presence of the DNA repair inhibitor Ara-C (cytosine arabinoside). **Results:** No significant difference was observed for MN frequency comparing nurses handling ANPDs (exposed subjects) and controls; no correlations were found between job seniority, age, smoking habits and MN rates. **Conclusions:** Concerning the aim of this study to evaluate the genotoxic risk arising from occupational exposure to ANPDs, statistically significant differences in MN rates in the subjects under study could not be determined.
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Key words: Antineoplastic drugs, Biological effect monitoring, Genotoxic risk, Micronucleus test, Occupational exposure

Antineoplastic drugs (ANPDs) are a heterogeneous group of chemicals that include alkylating agents (e.g., cyclophosphamide, melphalan, chlorambucil), antimetabolites (e.g., thioguanine, 5-fluorouracil, methotrexate), antibiotics (e.g., doxorubicin), mitotic spindle inhibitors (e.g., vincristine), hormones (e.g., diethylstilbestrol[1]), free radical generators (e.g., bleomycin) and topoisomerase inhibitors (e.g., irinotecan, etoposide). These drugs are often used in combination to achieve synergistic effects on tumor cells resulting from their differing modes of action. However, most if not all of these chemical agents are generally nonselective and, along with tumor cells, normal cells may undergo cytotoxic/genotoxic damage[1, 2].

Despite their therapeutic and beneficial effects in cancer patients with life-threatening conditions, both acute/short-term and chronic/long-term adverse effects are associated with ANPD treatment. Among the latter, several studies have pointed to the occurrence of secondary tumors in cancer patients as a result of their exposure to ANPDs[3]. Based on side effects in cancer patients and animal carcinogenicity data, as well as on results from *in vitro* studies, the International Agency for Research on Cancer (IARC) currently lists 11 agents and 2 combined therapies in clinical use in Group 1 (*i.e.*, human carcinogens,

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among which are busulfan, chlorambucil and cyclophosphamide), 12 in Group 2A (*i.e.*, probable human carcinogens, among which are cisplatin, etoposide and *N*-ethyl- and *N*-methyl-*N*-nitrosourea) and 11 in Group 2B (*i.e.*, possible human carcinogens, among which are bleomycins and mitomycin-C)[4].

Because of the widespread use of these mutagenic/carcinogenic agents in the treatment of cancer, concern has been raised about possible genotoxic hazards to medical personnel handling these drugs, such as pharmacists (during preparation), nurses (on administration), and physicians and nurses (in overall patient care). Following the first published data on the presence of mutagenic metabolites in urine from nurses occupationally exposed to ANPDs[5], data from several additional studies have confirmed the occurrence of work-environment contamination by ANPDs, with significant incorporation of trace amounts of these chemicals in hospital personnel, as revealed by indoor air, gloves and masks, surfaces or urine contamination with such agents[6]. Only a limited number of epidemiological studies have addressed occupational cancer risks related to handling of antineoplastic drugs. The main results reported in these studies are an increased risk of leukemia among oncology nurses and physicians occupationally exposed to ANPDs[7] and an elevated risk of cancer for long-term pharmacy personnel[8].

To minimize the risk of occupational exposure, guidelines for handling ANPDs, as well as safety recommendations, have been issued[9, 10]. For this type of occupational exposure, it is pivotal to assess the degree of genotoxic damage (*i.e.*, residual genotoxic risk), and several studies monitoring biological effects have been carried out. Widely used end points in genotoxic risk analysis in these professionals are sister-chromatid exchanges (SCEs), chromosomal aberrations, micronuclei (MNs) and DNA damage by comet assay. The cytokinesis-block micronucleus (CBMN) assay is one of the most frequently used test for biological effect monitoring in subjects occupationally exposed to ANPDs. However, the findings from different research groups are often conflicting.

No statistically significant differences in MN frequencies between hospital personnel occupationally exposed to ANPDs and nonexposed subjects were found in some studies[11–15], while, in others, higher frequencies of MN were found in either lymphocytes[16–19] or exfoliated buccal cells[15, 16] from healthcare workers preparing and/or administering ANPDs. The conclusions of these studies were often contradictory, probably because of uncertainties concerning exposure level[20] or because effective protective measures were differently adopted[21]. In a follow-up study of genotoxic hazards in pharmacists and nurses handling ANPDs carried out four years after a first study, the frequency of MN in the exposed nurses and control subjects was not found to be statistically different after improving working conditions[11].

The molecular epidemiology approach presented in this article was carried out as a part of a research project aimed at assessing the current level of exposure to ANPDs and DNA/chromosome damage as cancer predictive effects in occupationally exposed subjects. Overall, the research project consisted of an integrated chemical and biotoxicological approach for environmental and biological monitoring of exposure and cancer risks in healthy subjects occupationally exposed to ANPDs. The approach was based on monitoring procedures reported on the Italian guidelines[22], which include, besides methods for preventing exposure to ANPDs, monitoring recommendations. In particular, the guidelines provide guidance on the control of ANPD contaminations on surfaces and clothes by environmental monitoring (wipe and pad tests, respectively) and on the control of exposure by biological monitoring (concentrations of ANPDs in body fluids, usually urine), with both contamination and exposure depending on working practices and the frequency and adequacy of decontamination procedures.

In a previously published paper[23], we reported the results of DNA damage (assessed by the alkaline comet assay) in peripheral blood leukocytes of workers handling ANPDs; the levels of environmental exposure were evaluated by the wipe test and the degree of skin exposure by pads; the determination of urinary cyclophosphamide was also used as an indicator of biological uptake of specific ANPDs. On average, analysis of contamination in the work environment showed the presence of trace amounts of ANPDs, and urine samples were positive for cyclophosphamide in about 13% of the workers. The primary DNA damage levels were found to be statistically higher in exposed subjects than in controls, and this effect was particularly obvious in exposed subjects not using a mask.

Biological effect monitoring of exposure of pharmacists and nurses to ANPDs during their normal work routines was further assessed by the CBMN test in peripheral blood lymphocytes (PBLs), with blood samples for cytogenetic analyses being collected in the same monitoring session as for the comet assay. PBLs can be easily obtained by a minimum invasive route and are the cells most used in human biomonitoring studies. Further, lymphocytes display a half-life of 3–6 months and circulate throughout the body, integrating genotoxic events across body tissues. PBLs are thus generally considered as an appropriate surrogate target for the actual target tissues of geno-

toxic carcinogens, which are less readily available for biomonitoring investigations[24]. In this approach, the CBMN test was used because of its ability to detect both clastogenic (chromosome breakage) and aneugenic (spindle disruption) effects, allowing for a measure of genome damage that PBLs may have accumulated while circulating within the body in the quiescent phase. Moreover, it has recently been reported that the MN frequency in surrogate cells, such as lymphocytes, is a good predictor for cancer risk in human populations[25].

Compared with other cytogenetic assays, there are several advantages of quantifying MN, including speed and ease of analysis, and the fact that it does not require metaphase cells. Yet, this assay, like other cytogenetic assays, suffers from limitations. Chemically induced DNA damage in circulating lymphocytes can largely be repaired before the S-phase in stimulated cells, thus escaping fixation leading to chromosome breakage. To overcome the relative insensitivity of the assay to excision-repairable lesions, a modified protocol has been proposed. Incubation of lymphocytes with DNA synthesis inhibitors such as hydroxyurea, aphidicolin or cytosine arabinoside (Ara-C) blocks repair patch synthesis and thus converts excision-repairable lesions into MNs within one cell cycle[26]. In this study, the evaluation of MN frequency was carried out using the CBMN method in the absence or presence of the DNA repair inhibitor Ara-C.

Materials and Methods

Chemicals and media

All reagents used were of analytical grade. Acetic acid, Giemsa stain solution, methanol, potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4) and potassium phosphate monobasic (KH_2PO_4) were purchased from Carlo Erba Reagenti Srl, Milan, Italy. Cytochalasin-B, cytosine arabinoside (Ara-C) and phytohaemagglutinin (PHA), were obtained from Sigma-Aldrich Srl, Milan, Italy. Gibco® cell culture products, RPMI-1640 medium, *L*-glutamine, fetal calf serum (FCS) and antibiotics (*i.e.*, penicillin and streptomycin), were purchased from Invitrogen Srl, Milan, Italy. Conventional microscope slides and coverslips were supplied by Knittel-Glaser, Braunschweig, Germany. Vacutainer® blood collection tubes were from Becton Dickinson Italia SpA, Milan, Italy. Distilled water was used throughout the experiments.

Study population

The study included 48 health-care workers (41 females and 7 males) employed in a hospital in Perugia, Italy, regularly handling ANPDs (exposed subjects) and 50 subjects (38 females and 12 males) selected from workers in the administrative department from the same hospital, with no occupational contact with antineoplastic agents (nonexposed subjects). Participants were asked to fill in a questionnaire to provide details regarding demographic data (age, gender, etc.), lifestyle (smoking, alcohol consumption, etc.), health status (previous and present diseases, exposure to radiation therapy, etc.) and nature of occupation (working hours/day, years of service, use of protective measures, etc.); subjects with recent (less than 12 months) radiation exposure, either for therapeutic or diagnostic purposes, were not included in the study.

Approvals were obtained from the local ethics committee and health authorities. Exposed and nonexposed workers were informed about the aim and the experimental details of the study, and persons who met the required eligibility criteria were invited to participate and recruited on a voluntary basis. Written informed consent was obtained from all participating subjects.

Blood samples collection

Peripheral blood samples of the exposed and nonexposed subjects were collected from the antecubital vein by venipuncture into heparinized vacuum tubes. Samples from ANPD-exposed subjects were collected at the end of their work shifts. Blood samples from the two groups (exposed workers and controls) were collected weekly over the 6-month study period. An univocal serial code (corresponding to the questionnaire number) was assigned to each sample, before the vacuum tubes were dispatched to the laboratory for cytogenetic analysis. Processing of samples for exposed and control groups and microscope analysis were performed concurrently and without knowledge of subject identity with respect to exposure status.

Cytokinesis-block micronucleus test

The cytokinesis-block micronucleus (CBMN) test was performed according to the standard procedure[27], with minor modifications.

Lymphocyte cultures were established by adding 0.3 m*l* of whole blood to 4.7 m*l* of RPMI-1640 medium containing 20% heat-inactivated FCS, 1% *L*-glutamine, and antibiotics (100 IU/m*l* penicillin, and 100 μg/m*l* streptomycin). PHA (2%, from a 1.3 mg/m*l* stock solution) was added to stimulate cultures. Two independent cultures were set up for each subject and incubated in the dark at 37°C under 5% CO_2 humidified atmosphere conditions. After 44 hours of incubation, cytochalasin-B was added to the cultures at a concentration of 6 μg/m*l* to block cytokinesis of dividing cells. After a further 28 hours (whole culture time 72 hours), cells were harvested by centrifugation,

and lymphocytes were subjected to mild hypotonic treatment with 5 m*l* of a pre-warmed solution (75 mM KCl) to lyse erythrocytes. Cells were then prefixed in 5 m*l* of fresh ice-cold fixative solution (5:1 v/v methanol:acetic acid). Cell suspensions were then washed and fixed with 10 m*l* of fresh fixative for 1 hour on ice. Aliquots of cell suspensions were finally dropped onto precooled conventional microscope slides and air-dried. Slides were then stained for 5 minutes with 4% Giemsa in Sörensen's phosphate buffer (0.067 M Na_2HPO_4/KH_2PO_4; pH 6.8).

Microscope analysis was performed at 400 × magnification for detection and at 1,000 × magnification for confirmation on a light microscope (CX40, Olympus, Tokyo, Japan). All slides were analyzed blindly by an experienced scorer. For each individual, MNs were scored in 1,000 binucleated cells (500 cells per culture) with well-preserved cytoplasm, according to established criteria for identification of binucleated cells (e.g., two main nuclei, even partially overlapping, of approximately equal size, staining pattern and staining intensity) and scoring of MNs (e.g., small nuclei with diameter at least 1/3rd of main nuclei, separated from or marginally overlapping one main nucleus, with similar morphology and staining as the main nuclei)[27]. The MN frequency for each subject was expressed as MN/1,000 binucleated cells. Afterwards, the effects of exposure on cell proliferation were estimated by calculating the cytokinesis-block proliferation index (CBPI) according to the following formula[28]:

$$\mathrm{CBPI}=[M_{\mathrm{I}} + 2\ M_{\mathrm{II}} + 3\ (M_{\mathrm{III}} + M_{\mathrm{IV}})]\ /\ 500,$$

where M_{I} to M_{IV} represent the number of cells with one to four nuclei, with M_{III} and M_{IV} (*i.e.*, multinucleate cells) equally considered to be in their third cell cycle, and 500 corresponds to the total number of analyzed cells.

CBMN/Ara-C test

In order to increase the sensitivity of the test, a modified protocol was used (CBMN/Ara-C protocol)[26]. Two independent lymphocyte cultures per subject were established as above with the nucleoside analogous cytosine arabinoside (Ara-C) being added to the culture medium to a final concentration of 1 μg/m*l* in order to inhibit the gap-filling step in excision repair and convert excision repairable lesions into MNs within one cell cycle. Ara-C was removed by centrifugation after 16 hours, and cells were washed twice with complete medium and incubated for an additional 28 hours. Cytochalasin-B (6 μg/m*l*) was added to the cultures, and after an overall culture time of 72 hours, cells were harvested and analyzed as for the CBMN test.

Statistical analysis

Statistical analysis was performed with the SPSS 10 statistical package (SPSS, Chicago, IL, USA). Pearson's χ^2 test was used to evaluate differences of distributions for gender, age and smoking habits between exposed and nonexposed subjects. Seasonal trend of MN frequency over the course of the survey was tested using linear regression analysis. Differences between exposed and nonexposed subjects were analyzed by the nonparametric Mann-Whitney *U*-test. Two-sided p values <0.05 were regarded as statistically significant. Differences between subgroups were investigated using the Kruskal-Wallis *H* test. For significant results (p<0.05), to examine where the differences actually occurred, *post hoc* analysis was performed by running separate Mann-Whitney *U*-tests on the different combinations of related groups (multiple pairwise comparisons) with Bonferroni correction of the α in order to maintain the overall probability of a type I error at 0.05.

Significant results in the univariate analyses were included in a multiple linear regression model. Multivariate regression analysis was performed to examine the influence of exposure status, gender, age, smoking habits, occupational assignment, job seniority and personal protection as independent variables on the frequency of MNs.

Results

Demographic/occupational characteristisc

Exposed and control groups had comparable baseline characteristics, including age, gender and smoking habits (Table 1). The two populations were age-matched, and most of the subjects were females and nonsmokers both in the control and exposed groups; nonsmoker subjects had never been smokers or had stopped smoking at least 1 year earlier. Exposed subjects were divided according to their duties as follows, pharmacy technicians (n=6, involved in preparation of ANPDs), day hospital nurses (n=16, involved in administering drugs), ward nurses (n=19, involved in both preparation and administration of drugs), and attendants (n=8, personnel assigned to hospital waste disposal). The exposed nurses were gathered into three subgroups according to their job seniority: the majority of the subjects (31 individuals) had been exposed for less than 10 years, 13 worked for more than 10 and less than 20 years, and only 4 individuals had been exposed for more than 20 years. Pharmacy technicians, nurses and attendants were recommended to wear appropriate personal protective equipment (*i.e.*, disposable, single-use gowns, gloves and masks) whenever handling ANPDs or contaminated materials. All the exposed subjects wore gowns during their work shifts, and the great majority of

Table 1. Main characteristics of the study population

	Exposed	Controls	p
Subjects[a]	48	50	
Gender[a]			
Males	7 (14.6%)	12 (24.0%)	0.239
Females	41 (85.4%)	38 (76.0%)	
Age[b]			
Years	39.81 ± 9.56	36.56 ± 11.17	
<40 years[c]	29 (60.4%)	33 (66.0%)	0.567
≥40 years[c]	19 (39.6%)	17 (34.0%)	
Smoking habits[a]			
Nonsmokers	30 (62.5%)	36 (72.0%)	0.316
Smokers	18 (37.5%)	14 (28.0%)	
Occupational assignment[a]			
Pharmacy technicians	6 (12.5%)	—	
Day hospital nurses	16 (33.3%)	—	
Ward nurses	19 (39.6%)	—	
Attendants	7 (14.6%)	—	
Job seniority[a]			
≤10 years	31 (64.6%)	—	
11–20 years	13 (27.1%)	—	
>20 years	4 (8.3%)	—	
Personal protection[a, d]			
Gloves	2 (4.2%)	—	
Gloves + Mask	38 (79.2%)	—	
None	8 (16.7%)	—	

[a]Data are reported as the number of subjects (% between brackets). [b]Age is expressed in years and reported as the group mean ± standard deviation. [c]Cut-off defined according to the mean value (*i.e.*, 39.26 years) of the observed age distribution in the exposed subjects. [d]The environmental equipment for ANPD preparation in the pharmacy consisted of vertical air-flow cabinets.

them also wore gloves and mask, whereas a minority did not routinely wear gloves and/or mask. The environmental equipment for ANPD preparation in the pharmacy consisted of vertical air-flow cabinets. Table 2 summarizes the relative amounts of ANPDs handled over the 6 months study period (data obtained from questionnaires).

MN frequency - CBMN protocol

The analysis of cytogenetic damage, measured as MN frequencies in cytokinesis-blocked PBLs (CBMN protocol), is reported in Table 3. No significant difference was observed for MN frequency in comparison of nurses handling ANPDs (exposed subjects) and controls. To evaluate the effect of age as an effect modifier, MN frequency was calculated on the basis of a reference age value (40 years), corresponding approximately to the median value for age in both the exposed and control subjects (38 and 37 years, respectively). Gender and smoking habits were also considered. Gender, age and smoking habits were not associated with any increase in the frequency of MNs, either in the exposed or reference group. Females showed higher but not significant MN values when compared with males in both the exposed and control groups. Among the exposed subjects, no statistically significant differences were observed for the occurrence of MN in relationship to occupational assignment, job seniority and the use of personal protective equipment (*i.e.*, gloves and/or mask). The response to the PHA-mitogenic stimulus in lymphocyte cultures was evaluated by determining CBPI. No significant intergroup variations were observed for this parameter. Cytochalasin-B added after 44 hours of incubation, before the first mitotic wave for the great majority of cells, yielded about 65% of binucleated cells at 72 hours after PHA stimulation (65.9 and 66.4% for exposed and control subjects, respectively). No seasonal trend was seen during the 6-month study period (R^2=0.055).

Table 2. Relative amounts of antineoplastic drugs (ANPDs) handled over the 6-month study period (data obtained from questionnaires)

ANPDs	%	IARC[a]
5-Fluorouracil	26.4	Group 3
Cytarabine	17.0	Not listed
Cyclophosphamide	14.6	Group 1
Gemcitabine	14.4	Not listed
Ifosfamide	7.7	Not listed
Rituximab	4.6	Not listed
Methotrexate	2.5	Group 3
Etoposide	1.7	Group 1
Carboplatin	1.7	Not listed
Dacarbazine	1.6	Group 2B
Paclitaxel	1.4	Not listed
Trastuzumab	1.4	Not listed
Others[b]	5.0	—

Data are reported as the percentage (%) of each drug handled relative to the total amount of handled drugs (100%).
[a]Classification in the IARC Monographs. [b]Includes 33 ANPDs with individual relative amounts handled of less than 1%.

MN frequency - CBMN/Ara-C protocol

The average MN frequencies and CBPI values observed with the CBMN/Ara-C protocol are summarized in Table 4. As expected, there was an at least fivefold increase in MN frequency in cells cultured in the presence of Ara-C when compared with lymphocytes cultured according to the CBMN protocol. Statistical analysis indicated a significantly lower MN frequency in nurses exposed to ANPDs compared with control subjects (p=0.017; nonparametric Mann-Whitney U-test, two-sided). Within the groups (exposed or control), the differences in MN values in relationship to gender, age or smoking habits did not reach statistical significance. Female nurses exposed to ANPDs showed lower MN frequencies as compared with unexposed females. The lowest MN frequency was observed in exposed subjects with an age ≥ 40 years. CBPI values did not reveal significant intergroup differences in proliferation rates, although exposure of lymphocytes to Ara-C produced a general delay in cell cycle progression. As for the CBMN protocol, no seasonal trend was seen during the 6-month study period (R^2=0.027).

Table 3. Frequency of MNs per 1,000 binucleated lymphocytes and CBPI in nurses exposed to antineoplastic drugs and nonexposed subjects with respect of gender, age and smoking habits (whole population) and occupational assignment, job seniority and personal protection (exposed subjects)

	Exposed			Controls		
	n	MN	CBPI	n	MN	CBPI
Total	48	4.94 ± 1.67	1.93 ± 0.11	50	4.68 ± 1.49	1.96 ± 0.09
Gender						
Males	7	4.57 ± 0.89	2.00 ± 0.13	12	3.96 ± 1.59	1.94 ± 0.08
Females	41	5.00 ± 1.77	1.93 ± 0.11	38	4.91 ± 1.41	1.97 ± 0.10
Age						
<40 years	29	4.91 ± 1.19	1.94 ± 0.11	33	4.30 ± 1.33	1.98 ± 0.09
≥40 years	19	4.97 ± 2.24	1.92 ± 0.12	17	5.41 ± 1.55	1.93 ± 0.09
Smoking habits						
Nonsmokers	30	5.17 ± 1.81	1.93 ± 0.10	36	4.60 ± 1.45	1.97 ± 0.09
Smokers	18	4.56 ± 1.37	1.94 ± 0.13	14	4.89 ± 1.64	1.92 ± 0.10
Occupational assignment						
Pharmacy technicians	6	4.50 ± 1.55	1.87 ± 0.07		—	
Day hospital nurses	16	5.78 ± 1.96	1.93 ± 0.10		—	
Ward nurses	19	4.53 ± 1.20	1.93 ± 0.12		—	
Attendants	7	4.50 ± 1.76	2.01 ± 0.12		—	
Job seniority						
≤10 years	31	4.68 ± 1.19	1.95 ± 0.12		—	
11−20 years	13	5.23 ± 1.69	1.91 ± 0.09		—	
>20 years	4	6.00 ± 3.94	1.87 ± 0.05		—	
Personal protection						
Gloves	2	4.75 ± 1.77	2.09 ± 0.10		—	
Gloves + Mask	38	4.80 ± 1.43	1.93 ± 0.11		—	
None	8	5.62 ± 2.62	1.91 ± 0.10		—	

Data are reported as the group mean (± SD) of individual counts in subjects investigated with the cytokinesis-block micronucleus (CBMN) test. MN: micronuclei. CBPI: cytokinesis block proliferation index.

Table 4. Frequency of MNs per 1,000 binucleated lymphocytes and CBPI in nurses exposed to antineoplastic drugs and nonexposed subjects with respect of gender, age and smoking habits (whole population) and occupational assignment, job seniority and personal protection (exposed subjects)

	Exposed			Controls		
	n	MN	CBPI	n	MN	CBPI
Total	47	20.37 ± 4.33*	1.78 ± 0.15	50	23.25 ± 6.06	1.83 ± 0.11
Gender						
Males	7	23.36 ± 5.26	1.82 ± 0.07	12	21.92 ± 5.81	1.83 ± 0.09
Females	40	19.85 ± 4.00	1.76 ± 0.16	38	23.67 ± 6.15	1.83 ± 0.12
Age						
< 40 years	28	21.59 ± 4.77	1.79 ± 0.12	33	23.41 ± 6.17	1.82 ± 0.10
≥ 40 years	19	18.58 ± 2.86	1.74 ± 0.19	17	22.94 ± 6.00	1.86 ± 0.13
Smoking habits						
Nonsmokers	30	20.15 ± 4.09	1.75 ± 0.15	36	23.93 ± 6.30	1.84 ± 0.12
Smokers	17	20.76 ± 4.83	1.79 ± 0.15	14	21.49 ± 5.18	1.81 ± 0.09
Occupational assignment						
Pharmacy technicians	6	19.00 ± 4.34	1.71 ± 0.10		—	
Day hospital nurses	15	22.23 ± 4.07	1.82 ± 0.10		—	
Ward nurses	19	19.29 ± 4.33	1.71 ± 0.19		—	
Attendants	7	20.50 ± 4.37	1.86 ± 0.09		—	
Job seniority						
≤ 10 years	30	21.09 ± 4.71	1.79 ± 0.13		—	
11−20 years	13	19.19 ± 3.79	1.70 ± 0.20		—	
> 20 years	4	18.75 ± 1.04	1.83 ± 0.08		—	
Personal protection						
Gloves	2	21.75 ± 6.01	1.87 ± 0.09		—	
Gloves + Mask	38	20.59 ± 4.60	1.77 ± 0.14		—	
None	7	18.79 ± 1.84	1.73 ± 0.21		—	

Data are reported as the group mean (± SD) of individual counts in subjects investigated with the CBMN/Ara-C test.
* Two-sided p value < 0.05 *vs*. corresponding controls; nonparametric Mann-Whitney *U*-test. MN: micronuclei. CBPI: cytokinesis block proliferation index.

The results obtained with the CBMN/Ara-C protocol were further analyzed by multiple linear regression analysis. The analysis was first done on the whole population and thereafter on subjects stratified by occupational exposure. The analysis did not show statistically significant correlations between the independent variables exposure, gender, age, smoking habits (exposed *vs*. controls), occupational assignment, job seniority, personal protection (exposed subjects) and MN frequencies (data not shown).

Discussion

Nurses handling ANPDs are exposed to a variety of chemicals such as alkylating agents, antimetabolites, antibiotics, mitotic spindle inhibitors, hormones, free radical generators and topoisomerase inhibitors in unknown nontherapeutic concentrations and in mixtures with potentially interacting mechanisms. In this paper, we report the results of a biological effect monitoring study performed to complete the assessment of genetic effects of exposure to environmental genotoxins in a group of health-care workers occupationally exposed to ANPDs. The frequency of MNs was evaluated in PBLs of subjects also monitored for primary DNA damage by the comet assay[23]. The alkaline comet assay detects primary DNA lesions (e.g., DNA double– and single–strand breaks, alkali–labile sites such as apurinic/apyrimidinic sites and incomplete excision repair sites) present in leukocytes (or lymphocytes) at the time of blood sampling[29]. The CBMN test allows for a measure of genome damage that may have accumulated while lymphocytes circulate within the body in the quiescent phase. Further, lymphocytes display a half-life of 3−6 months and circulate throughout the body, integrating genotoxic events across body tissues[30].

The results indicated no exposure-related excess of MNs in exposed subjects compared with controls, despite the occurrence of detectable environmental

contamination by 5-fluorouracil (5FU) and cytarabine (CYT) on selected surfaces (wipes) and on the exposed nurses' clothes (pads)[23]. Similar to our findings, negative results for MN induction were reported in other investigations on workers exposed to ANPDs[11–15]. As reported previously[23], health-care workers handling ANPDs were evaluated for genotoxic damage in peripheral blood leukocytes, and primary DNA damage was evaluated by applying the comet assay for detecting early biological effects of DNA-damaging agents (*i.e.*, antineoplastic drugs). We found higher DNA migration extents in the exposed subjects when compared with the controls. The presence of primary DNA damage revealed previously by comet assay testing of peripheral blood leukocytes from health-care workers exposed to ANPDs and the negative results reported in the present study could suggest that the CBMN assay may lack the sensitivity required to detect the effects produced by low-level exposures to genotoxic agents. On the other hand, the comet assay and the CBMN test detect genotoxic effects caused by different mechanisms; the comet assay identifies still reparable injuries such as single- and double-stranded DNA breaks, alkali labile lesions (*i.e.*, apurinic/apyrimidinic sites) that are converted to strand breaks under alkaline conditions and single-strand breaks associated with incomplete excision repair sites, whereas the CBMN assay detects injuries that survive at least one mitotic cycle and reflect unrepaired, fixed DNA damage. It might be possible that occupational exposure to low levels of ANPDs induced primary DNA damage (detected by the comet assay), with damage efficiently repaired and not fixed as cytogenetic alterations (detected by CBMN test).

The fact that smoking habits did not affect the frequency of MN, either in the exposed or in the control subjects, was not unusual. In fact, even when concomitant exposure to genotoxic xenobiotics is taken into account, according to a recent review of literature data, smokers will not show any increase in MN frequency when compared to nonsmokers[31]. Also gender and age were not associated in this study with any increase in the frequency of MNs, either in the exposed or reference group. Higher frequencies of MNs have often been reported in females than in males[31]. In our study, gender exerted a slight influence on the occurrence of MNs in both groups; however, differences between averaged MN frequencies did not reach statistical significance, likely because of the low number of males in both groups under investigation. It has been reported that the number of MNs in PBLs increases with age in both males and females[31]. In our study, MN frequencies tended to rise with age, although not significantly, only in the control group, probably because the age ranges were very similar (23–59 and 19–59 years, for exposed and controls, respectively), with about 2/3 of the subjects less than 40 in both groups.

The significantly lower MN frequency in nurses exposed to ANPDs compared with control subjects observed with the CBMN/Ara-C protocol was an unexpected result. To the best of our knowledge, this is the first study applying the CBMN/Ara-C protocol in this occupational area, and thus it is not possible to make any comparison with other results. This unexpected outcome could be perhaps explained in terms of adaptive response. In fact, it has been reported that exposure to low doses of radiomimetic chemicals, alkylating agents, cross-linking agents or ionizing radiation can lead to a decreased susceptibility of cells to genotoxic agents[32]. DNA damage induced in circulating lymphocytes is likely to persist and accumulate because of the limited excision repair activity in quiescent (G_0) cells[33]. However, following mitogen stimulation, repair proficiency is rapidly restored[34], and DNA damage can largely be repaired before the S-phase, thus escaping fixation as a permanent alteration in DNA, particularly in cells with an induced adaptive response[35]. An adaptive response in human lymphocytes from subjects with occupational exposure to genotoxic agents has been reported for several genotoxic endpoints, including chromosome aberrations[36] and MNs[37], and could be relevant to our findings.

Taking the overall results of our two monitoring studies into consideration, we have observed that: 1) environmental (surface/clothing) contamination levels were found to be similar to those reported for other Italian hospitals[38], even with a lower number of positive samples; 2) a very low number of urine samples showed detectable concentrations of the marker compound (*i.e.*, cyclophosphamide), a result in agreement with recent findings indicating surface contamination as not necessarily correlating with positive urine samples[38], probably because of suboptimal sensitivity of the adopted assays to detect lower concentrations of the drugs in urine; 3) an increased extent of primary DNA damage was observed in exposed subjects handling ANPDs, a positive finding in line with the results from several other studies[14, 17, 19, 39, 40] and 4) no significant differences were found for MN frequencies when comparing nurses handling ANPDs and controls (CBMN protocol), in line with previously published studies[11–15], but an adaptive response in human lymphocytes from subjects with occupational exposure to ANPDs (CBMN/Ara-C protocol) was observed.

The most sensitive biomarker seems to be the comet assay. The comet assay is a rapid, simple and very sensitive test able to reveal early, still repairable

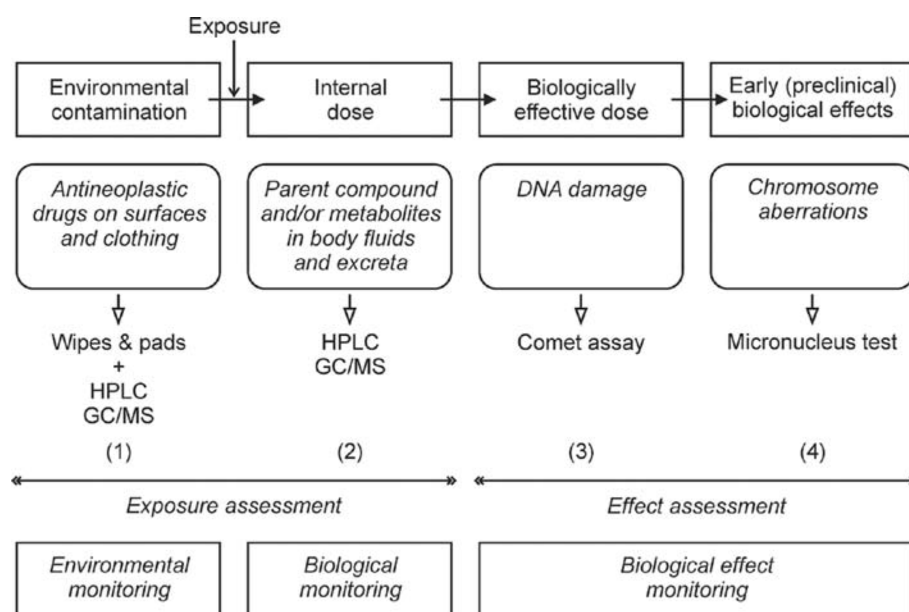

Fig. 1. Proposed model for monitoring occupational exposures to genotoxic compounds in health-care settings.

DNA damage, and can therefore provide useful information on early effects induced by occupational exposure to low doses of complex mixtures of genotoxic compounds, such as ANPDs. Based on these results, Fig. 1 depicts an integrated chemical and biotoxicological approach for environmental, biological and biological effect monitoring of exposure to genotoxic ANPDs.

The Italian guidelines[22] aimed at preventing or at least minimizing occupational exposure to ANPDs include not only methods for preventing exposure (engineering controls, administrative and work practice controls and personal protective equipment), but also monitoring recommendations. In particular, the guidelines provide guidance on the control of ANPD contaminations on surfaces and clothes by environmental monitoring (step 1 in Fig. 1) and on control of exposure by biological monitoring (concentrations of ANPDs in body fluids, usually urine) (step 2 in Fig. 1), with both contamination and exposure depending on working practices and frequency and adequacy of decontamination procedures. Approaches similar to that proposed by the Italian guidelines can be obtained from many other guidelines or documents issued to minimize the risk of occupational exposure during handling of ANPDs. Analytical methods for routine monitoring of occupational exposure to antineoplastic drugs (e.g., high performance liquid chromatography, gas chromatography/mass spectrometry) are very sensitive and specific. Furthermore, the advantage of biological monitoring is its ability to measure the total uptake of ANPDs by all routes of exposure. Nevertheless, for both environmental and biological monitoring, testing is generally limited to one or very few agents that are considered as model compounds. In the proposed approach, monitoring of genotoxic risks should be performed by combining environmental and biological monitoring with procedures for biological effect monitoring (primary DNA damage and chromosome damage) (steps 3 and 4 in Fig. 1). In the integrated chemical/biotoxicological approach proposed, the use of biomarkers measuring changes in cellular or molecular endpoints (e.g., DNA and/or chromosome damage) will allow us to apply a more complete approach focusing not only on environmental and biological monitoring but also on biological effect monitoring using genotoxicity biomarkers. In this context, the comet assay represents a highly sensitive technique for detecting low levels of DNA damage in individual cells[29] and could be proposed to be used to accurately monitor interaction of ANPDs with DNA (biomarker of biologically effective dose). Among biotoxicological tests, the frequency of MNs in PBLs is recognized to be a predictor of cancer risks in human populations[25], and because of its ability to detect both clastogenic (e.g., chromosome breakage) and aneugenic (e.g., spindle disruption) effects, it could have a role in occupational health surveillance programs for workers occupationally exposed to ANPDs to monitor long-term exposure effects (biomarker of early/preclinical biological effects).

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