Comparative mouse micronucleus evaluation in bone marrow and spleen using immunofluorescence and Wright’s Giemsa

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Summary

Bone marrow and spleen toxicity, clastogenicity and aneugenicity were analyzed in the CD1 mouse using an antikinetochore antibody (AKA) procedure (Krishna et al., Mutation Res., 282, 159–169, 1992). Further, to verify the fluorescence micronucleus (MN) analysis, additional slides were stained with Wright’s Giemsa and results were compared. 5 mice per sex were treated with cyclophosphamide (CP) (40 mg/kg) or vincristine (VC) (0.1 or 0.2 mg/kg). Slides were prepared 24 h postdose using a column fractionation procedure. Per animal, 400 total erythrocytes (TEs) for toxicity and 2000 polychromatic erythrocytes (PCEs) for MN per tissue were analyzed. In the fluorescent method, the clastogen, CP, produced MNPCEs predominantly devoid of kinetochores (K) and the aneugen, VC, produced mostly MNPCEs containing K. The MNPCE frequency did not differ significantly between tissues; however, it differed statistically between sexes. On an overall basis, spleen had significantly lower PCE to TE ratios compared to bone marrow. In general, CP and VC caused a small, but statistically significant decrease in PCE frequencies compared to controls, suggesting possible toxicity to these tissues at the given doses. The data on Wright’s stain indicated that the proportion of PCEs and MNPCEs in general, were comparable to those using fluorescent stain. This study further confirms the usefulness of an AKA-staining technique in a multiple genetic endpoint evaluation under a single set of microscopic conditions.

In vivo assessment and identification of aneuploidy is one of the important phases of genotoxicity evaluation (Cimino et al., 1985; Liang and Satyaprakash, 1985; Dellarco et al., 1986; Onfelt, 1986; Miller and Adler, 1989; Vanderkerken et al., 1989; Wang Xu and Adler, 1990; Adler et al., 1991; Parry and Parry, 1991). Aneuploidy-inducing agents (aneugens) generally produce micronuclei containing whole chromosome(s) and clastogens produce micronuclei containing predominantly acentric fragments (Parry and Parry, 1987; Rizzoni et al., 1989). The use of antikinetochore antibody (kinetochore-labeling

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procedure) can distinguish the mechanisms of micronucleus production.

A few investigators have reported the use of antikinetochore antibody under in vivo conditions to differentiate aneugens from clastogens (Gudi et al., 1990; Miller and Adler, 1990; Miller et al., 1991). In these studies, the mouse bone-marrow micronucleus assay was utilized and micronuclei were scored in total erythrocytes. However, in the routine acute micronucleus assay, the micronuclei are evaluated in polychromatic erythrocytes (PCEs). The prior studies could not differentiate between PCEs and normochromatic erythrocytes (NCEs). Gudi et al. (1990) reported the use of whole bone marrow for standard smear preparation. The presence of nucleated cells in the whole bone marrow interferes with accurate evaluation of micronuclei because of background fluorescence.

Recently, we made an attempt to improve the antikinetochore-antibody approach described earlier for the in vivo mouse bone-marrow micronucleus assay (Krishna et al., 1992). To eliminate nucleated cells and to minimize background fluorescence, a cellulose column cell fractionation method was utilized. Further, the sequential treatment of slides with CREST serum (antikinetochore antibody), fluorescein-conjugated goat-antihuman and swine-antigoat antibodies, and propidium iodide was used. This method helps differentiate PCEs from NCEs and facilitates analysis of micronuclei for the presence or absence of kinetochore(s) in PCEs under one filter setting. This procedure is easy and can be adapted for routine testing. A similar approach has been recently used by Gudi et al. (1992).

In the present study, to further validate this technique, vincristine sulfate (an aneugen), and cyclophosphamide (a clastogen) were used. The study was performed in both male and female mice in order to assess differences between sexes. Also, for comparative purposes, in addition to bone marrow cells, spleen cells were utilized as spleen cells have been used in a variety of genotoxicity studies (Krishna et al., 1987, 1988, 1990b). Further, to verify the validity of fluorescence micronucleus analysis, additional slides were stained with standard Wright’s Giemsa and PCE and MNPCE frequencies were assessed. The results of these studies are reported here.

Materials and methods

Chemicals. Cyclophosphamide (CAS No. 50-18-0) was obtained from Sigma Chemical Company, St. Louis, MO. Vincristine sulfate was purchased from Aldrich (Milwaukee, WI). Polyoxyethylene sorbitan monolaurate (Tween 20) and propidium iodide were obtained from Sigma. The dose selection for these test agents was based on the literature (Gudi et al., 1990; Mavournin et al., 1990; Krishna et al., 1990b, 1992) and our preliminary studies. A kinetochore positive control serum for the antinuclear antibody test was purchased from Antibodies, Inc. (Davis, CA). This primary antibody is highly specific for a centromere-associated epitope which is assumed to be the kinetochore. The fluoresceinated goat antihuman and swine antigoat γ-globulins were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Animals. 6–8-week-old CD-1 male and female mice obtained from Charles River Breeding Laboratories (Portage, MI), were housed individually in stainless steel wire mesh cages and acclimatized for 1 week before dosing. The animals were observed before initiation of the study to ensure that they were healthy. Only animals found to be in a clinically acceptable condition were assigned to the study. During the study, food (Purina Certified Rodent Chow® 5002) was offered ad libitum in stainless steel food containers. Water was supplied ad libitum by an automatic watering system. An automatic timer provided the animal room with an alternating 12-h cycle of light and darkness. The temperature (68–78°F) and relative humidity (30–75%) in the animal room were within specified ranges at all times during the study. Each experimental group consisted of 5 animals per sex.

Chemical treatment. Single doses of vincristine (0.1 and 0.2 mg/kg) or cyclophosphamide (40 mg/kg) were injected intraperitoneally at a dose volume of 10 ml/kg. Saline (0.9% NaCl) at a dose volume of 10 ml/kg served as control.
Animal sacrifice, bone-marrow and spleen cell isolation and slide preparation. Bone-marrow cells for micronucleus assessment were isolated and slides were prepared as described by Schmid (1975, 1976), Ashby and Mohammed (1986), Romagna and Staniforth (1989), and Krishna et al. (1990a, b, 1992). Spleen was also isolated from the same animal by opening the abdominal cavity, transferring the spleen into a tube containing 3 ml FBS, rupturing with a spatula, and removing debris. The cell suspension was placed on a cellulose column and washed using Hanks’ balanced salt solution (HBSS, without phenol red). The eluant was spun at 800 x g for 10 min and the supernatant discarded. The pellet was resuspended in an appropriate amount of HBSS and slides were prepared using a cytocentrifugation method. 6 slides per tissue per animal were made and 4 slides per tissue per animal were fixed in methanol and stained using an immunofluorescent method described briefly below (Krishna et al., 1992). The remaining 2 slides were stained with Wright’s Giemsa.

Antibody-labeling procedure
Slides were soaked in PBS–0.01% Tween 20 for 4 min, after which the excess fluid was drained (care was taken to keep slides moist throughout the procedure). The antikinetochore–antibody solution was diluted with an equal volume of PBS, and 50 μl of this solution was placed on each slide. The slides were coverslipped and placed in an incubator (37°C, humidified) for 1 h. Following incubation, the coverslips were removed and slides were rinsed in PBS for 3 min with frequent agitation. After a second rinse with fresh PBS, slides were rinsed with PBS–0.1% Tween 20 for 2 min and the excess fluid was drained. A 50-μl aliquot of fluoresceinlabeled goat–antihuman antibody previously diluted 1:20 with PBS was placed on each slide. The slides were coverslipped, incubated for 50 min, rinsed, and excess fluid was drained as before. These slides were labeled again with 50 μl of a second antibody, fluoresceinlabeled swine–antigoat antibody, previously diluted 1:20 in PBS. Slides were coverslipped and incubated for 50 min. Following incubation, the slides were rinsed with two changes of PBS and excess fluid was drained. Slides were placed in 4 μg/ml propidium iodide solution (in PBS) for 40 sec and rinsed once in PBS for 1–2 sec. Finally, 2 drops of antifade solution were placed onto each slide and the slides were coverslipped. The slides were blind coded and either scored immediately or stored in the refrigerator (in dark) up to a week until scoring was completed.

Scoring procedure
For scoring, an American Optical microscope equipped with epifluorescence was used. The slides were scored under blue excitation (440–490 nm) with a barrier filter combination at 520 nm. In this method, PCEs stain dark red, NCEs stain green, chromosome(s)/fragment(s)/micronuclei stain orange, and kinetochores stain yellow. For micronucleus analysis in PCEs, 2000 PCEs per animal per tissue were analyzed. The criterion for scoring micronuclei in PCEs as kinetochore negative (K−, absence of yellow spot in the micronucleus), or positive (K+, presence of one or more yellow spots in the micronucleus) was described previously (Krishna et al., 1992). The morphological criteria regarding micronucleus scoring in a PCE were similar to earlier studies (Schmid, 1975, 1976; Heddle et al., 1983; Ashby and Mohammed, 1986; MacGregor et al., 1987; Mourin et al., 1990). Slides stained with Wright’s Giesma were also blind coded and 2000 PCEs were evaluated per tissue per animal for MN-PCEs. To evaluate potential drug toxicity to the bone marrow and spleen, proportions of PCEs were counted based on 400 total erythrocytes (TEs) per animal per tissue.

Statistical analysis of data
The square roots of arcsine transformed data were used to improve the homogeneity of variance and to better comply with the normality assumption. The analysis of variance procedure was used to compare each treatment group with vehicle control group. Separate statistical analyses were conducted for each sex, taken separately and combined and between staining procedures. The primary measures were the frequency of PCEs in the erythrocyte population, the frequency of MN-PCEs in the PCE population, and the proportion of K+ and K− MN-PCEs (where appropriate).
Results

Antikinetochore antibody specificity to the centromeric region, the differentiation of PCEs and NCEs, and the appearance of kinetochore(s) within the micronuclei of mouse bone marrow and spleen erythrocytes using the antikinetochore antibody technique described in the Materials and methods section is shown in Fig. 1. Fig. 1a shows a mitotic cell (1000 X, V79 Chinese ham-

Fig. 1. Photomicrographs of cells stained with antikinetochore antibody, fluoresceinated antibodies, and propidium iodide. (a) A metaphase cell showing centromere specific staining of CREST serum (V79 Chinese hamster lung cell chromosomes, for example). (b) A microscopic field showing cellulose-column fractionated cytospun cyclophosphamide-treated mouse bone-marrow polychromatic erythrocyte (PCE) and normochromatic erythrocyte (NCE) differentiation; PCEs with kinetochore negative micronuclei (arrows). (c) A microscopic field showing vincristine-treated spleen PCEs with kinetochore positive micronuclei (arrows).
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Values are expressed as mean ± SD of 5 (M, F) or 10 (M + F) animals.
C, control; CP, cyclophosphamide; VC, vincristine; PCEs, polychromatic erythrocytes; MNPCEs, micronucleated PCEs; TEs, total erythrocytes; K−, kinetochore negative; K+, kinetochore positive.

* Significantly different from controls, p < 0.05; all bone-marrow PCE values were significantly different from spleen PCE values, p < 0.01.
Fig. 2. (a) The process of erythropoiesis in vivo; (b) the mechanism of micronucleus formation in the polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). Also, classification of kinetochore positive (K+) and kinetochore negative (K−) erythrocytes. N, nucleus; PEB, proerythroblast; MN, micronucleus.
ster lung cell, for example; photo taken in a preliminary study) which was stained with CREST serum demonstrating antikinetochore antibody specificity. Fig. 1b shows cyclophosphamide-treated bone-marrow PCE and NCE differentiation under a microscopic field (1000 ×) and a MNPCE. The micronucleus in the PCE is K−. Fig. 1c shows vincristine-treated spleen cells with 3 MNPCes which are K+ and one of these MNPCes contains 2 kinetochore spots in a micronucleus. These photos, taken under one filter-setting, demonstrate the usefulness of the technique for routine testing.

Using this technique in mice, saline controls and 2 test agents were evaluated for the percent PCEs in the bone marrow and spleen erythrocyte population, the frequency of MNPCes, and the presence of kinetochore spots in the MNPCes (Table 1). In the bone marrow, the saline controls contained 1.2 and 1.3 MNPCes per 1000 PCEs in males and females, respectively. Among these cells (combined sex), 48% were K− and 52% were K+. Spleen cells also contained similar frequencies of MNPCes as well as K− and K+ MN.

The clastogen, cyclophosphamide at the dose tested, clearly increased MNPCE frequencies in both sexes. For cyclophosphamide essentially all of the MNPCes were K− (98% in bone marrow and 95% in spleen, based on combined sex). For cyclophosphamide, in the bone marrow, a slight but statistically significant decrease in % PCEs was observed in females. However, the percent PCEs in cyclophosphamide-treated animals did not differ significantly from control values in bone marrow (males) and spleen (males and females separately).

The aneugen, vincristine, also clearly induced dose-related increases in MNPCE frequencies in both sexes and in both tissues. In bone marrow, approximately 89% of MNPCes were K+ for both doses (0.1 or 0.2 mg/kg, combined sex) tested. Similar frequencies of K− and K+ MNPCes were also noted in spleen cells. For vincristine, percent PCEs were in general slightly lower than controls in both sexes and differed significantly. On an overall basis, the percent PCEs were lower in spleen compared to bone marrow in all animals (including controls) and drug-induced MNPCE frequencies were significantly different between sexes. The PCE and MNPCE frequencies following various treatments in the Wright's Giemsa method are also shown in Table 1. The data on Wright's stain indicated that the proportion of PCEs and MNPCes in general, was comparable to those of fluorescent stain and was not significantly different.

Discussion

The process of erythropoiesis and the mechanism of micronucleus formation in vivo are shown in Figs. 2(a) and (b). In the adult mouse, both bone marrow and spleen are hemopoietic organs (Bradsky et al., 1966; Bannerman, 1983; Shindo et al., 1983). In the hemopoietic organs, the stem cells form the basis of erythropoiesis. Generally, the stem cells undergo proliferation and maturation stages. In the proliferation stage, cells continue to divide at which time a given test agent may act on dividing cells. Test agents may produce chromosome damage such as breaks and exchanges and may also cause spindle dysfunction depending on mechanism of action. These anomalies may lag behind in the cell division process and may not become part of the daughter nuclei and may eventually form micronuclei. In the maturation stage, the newly formed cells will lose their main nuclei (enucleation) and still contain RNA. These are termed PCEs (young erythrocytes). By some mechanism, the majority of the micronuclei escape from the process of enucleation and generally remain in PCEs and micronuclei are generally analyzed in such cells. These cells with time, lose RNA and contain primarily hemoglobin and become NCEs (mature erythrocytes). These two types of erythrocytes can be seen in bone marrow and spleen and they stain differentially. Later, and on a needed basis, these cells move into the blood compartment. The kinetochore-labeling procedure can distinguish the mechanism of either a clastogen-induced damage (primarily chromosome breakage and the absence of kinetochores in the micronucleus) or an aneugen-induced spindle dysfunction (primarily lagging chromosome(s) and the presence of kinetochores(s) in the micronucleus) in the erythrocytes.
In the routine micronucleus assay, bone marrow has been used as a target organ; however, it would be of interest if an additional tissue/organ such as the spleen can also be used for micronucleus analysis. This may help decrease the false positives and false negatives and clarify marginal results with relatively less effort. Previously, we developed a method which can be used to evaluate clastogenicity, aneugenicity, and toxicity simultaneously in the mouse bone-marrow micronucleus assay (Krishna et al., 1992). In the present study, we have further validated such an approach and have extended such an evaluation to spleen tissue. Spleen tissue has been used to analyze micronuclei previously (Shindo et al., 1983; Krishna et al., 1990b). Also, several recent cytogenetic studies have used spleen lymphocytes to evaluate genotoxicity under in vivo and in vitro conditions (Krishna et al., 1986, 1987, 1988; Tucker et al., 1986; Palitti et al., 1982; Solern-Niedziela et al., 1989). Therefore, in this study, spleen PCEs were used to supplement the bone marrow results and to determine the effects of test agents on two different tissues/sources of PCEs.

Previously we reported that the cellulose column bone-marrow cell fractionation procedure provided excellent preparations for the manual assessment of clastogenicity, aneugenicity and toxicity simultaneously in mouse bone marrow (Krishna et al., 1992). The nucleated cell population, including cell debris, was essentially eliminated as a result of column fractionation and the detection of micronuclei was facilitated due to the flattening of cells resulting from cytocentrifugation. Also, in the immunofluorescence microscopy, this approach helps minimize/remove the background fluorescence. In the present study this approach has been extended to spleen cells. An evaluation of such a method in spleen is more significant since in an adult normal mouse spleen contains fewer PCEs (~10%) than bone marrow (~15%) in relation to total cell composition. Thus, it is advantageous to eliminate nucleate cells by the column and to have PCEs and NCEs for easy scoring. In the immunofluorescent method the quality of slides, for example PCE and NCE differentiation, may differ depending on the test compound. In the present study, the cells from cyclophosphamide-treated animals were clearly differentiated (Fig. 1b). However, the cells from vincristine-treated animals, although differentiated, had slightly different color (Fig. 1c). All of these slides were processed and stained in one batch. These differences may be due to the differential effects of test drugs on the cellular proteins which in turn might affect staining quality. In the present study, however, this did not affect scoring.

The validation results presented in this paper indicate that this methodology exhibits considerable specificity in distinguishing aneuploidy-inducing agents from clastogenic agents. Based on the results in this study, cyclophosphamide appears to be a pure clastogen and vincristine an aneugen. Similar results have been reported for these drugs using various methods in bone marrow (Tinwell and Ashby, 1991; Gudi et al., 1992). The magnitude of total MNPCE response and the response of K + and K − micronuclei were, in general, comparable in both tissues and in both sexes for each test drug separately. This suggests a relatively similar sensitivity of these two tissues for cyclophosphamide and vincristine. A slight but statistically significant difference in MNPCE response between sexes was noted; however, this difference may not be biologically significant.

Differentiation of PCEs and NCEs in this study made it possible to also evaluate toxicity of test drugs to the bone marrow and spleen. Assessment of bone-marrow and spleen toxicity is an essential endpoint in the micronucleus assay. Cyclophosphamide produced a slight but statistically significant decrease in percent PCEs in females but not in males. Cyclophosphamide, at 40 mg/kg, has been routinely used as a positive control compound in the micronucleus assay in our laboratory with no or minimal toxicity (Krishna et al., 1990b). Vincristine, at 0.1 and/or 0.2 mg/kg produced some toxicity. However, in an earlier study, Tinwell and Ashby (1991) did not notice any toxicity up to 0.5 mg/kg following i.p. treatment of male mice with vincristine.

The proportion of PCEs in relation to TEs is significantly higher in the bone marrow compared to spleen, indicating tissue specificity with reference to hemopoietic function between these two
organs. Estimates of the number of stem cells in different blood-forming tissues of normal mice have been obtained by transplanting known numbers of cells into lethally irradiated mice. Using stem cells, it has been reported that normal bone marrow contains approximately ten colony forming units (CFU) per $10^5$ cells, and normal spleen approximately one CFU per $10^5$ cells (Russell and Bernstein, 1966). Thus, indicating a 10:1 proportionality between bone marrow and spleen in blood formation. This is in agreement with a prior study by Shindo et al. (1983) and further suggests that bone marrow is the primary organ in the hemopoietic process; however, such a process may be secondary to the spleen (Brodsky et al., 1966). The partitioning of hemopoietic production between marrow and spleen varies according to stage of development and circumstances. Marked enlargement of spleen occurs during pregnancy. Other stimuli which increase splenic erythropoiesis include bacterial infections, bacterial toxins, graft-versus-host reaction, and hemolytic anemia (Bannerman, 1983).

In this study, the comparative analysis of fluorescent stain with that of Wright's indicated comparable results. This suggests that fluorescent method of analysis of micronucleus is reliable. Further, it was found that keeping fluorescent stained slides up to a week at 4°C during the course of scoring did not significantly alter the quality of slides.

In summary, the data reported here further demonstrates the usefulness of the antikinetochore antibody staining technique in the mouse micronucleus assay for routine testing to simultaneously assess toxic, clastogenic, and aneugenic effects in both bone marrow and spleen.

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