The Micronucleus Test as an *in Vivo* Cytogenetic Method

by Robert C. Miller*

Introduction

Methods are currently being explored which would permit the application of cytogenetic procedures such as metaphase and anaphase analyses to *in vivo* mammalian systems in order that due consideration may be given to metabolic activation or detoxification reactions which might affect the mutagenic or clastogenic (chromosome breaking) potential of environmental agents under study. It has been recommended (*1*) that such *in vivo* cytogenetic methods be employed in mutagenicity testing programs which are being designed to evaluate mutagenic potentials and risk-benefit ratios of a wide variety of drugs and other chemical agents when human exposure is involved.

Metaphase chromosome analysis has been adapted readily to *in vivo* testing of chemical mutagens (*2–5*). More recently, Palmer, Kelly-Garvert, and Legator (*6*) have described a potentially useful method for *in vivo* collection of anaphase figures from rat bone marrow cells which involves the application of a mitotic arresting agent (colcemide, CIBA, New Jersey) *in vivo*, followed by a short-term *in vitro* incubation (1–1½ hr) in the absence of colcemide to reconstitute spindle fibers and initiate anaphase movement. Preliminary studies with the *in vivo* anaphase method in our laboratory have revealed a number of technical limitations which have restricted its value for rapid screening of potential clastogenic agents; the most troublesome of these being the extremely small size of the anaphase figures obtained, necessitating time-consuming scanning of slides using oil immersion optics, and the relatively high baseline aberration rate of approximately 10% in preparations from untreated control animals, leading to serious questions regarding the sensitivity of the procedure.

The purpose of this paper is to outline work being done on an alternative method for scoring *in vivo* clastogenic effects of potential mutagens which may provide a method for the rapid screening of large numbers of chemical compounds: the bone marrow "micronucleus test" as modified by Schmid and his co-workers at the University of Zurich. (*7*).

Rationale

The micronucleus test (*7*) affords a procedure for the detection of aberrations involving anaphase chromosome behavior utilizing a particularly useful cell type, the bone marrow erythroblast. The test is based on the formation of "micronuclei" from particles of chromatin material which, due to chromosome breakage or spindle dysfunction, do not migrate to the poles during anaphase and are not incorporated into the telophase nuclei of the dividing cell. Such chromatin fragments, or even whole chromosomes in the case of chromosome lag, re-

*Institute for Medical Research Camden, New Jersey 08103.

sult in the formation of one or more small satellite nuclei in the cytoplasm of the daughter cells. In animals treated with known clastogenic agents such as 2,3,5-tris-(ethylenimino)-1,4-benzoquinone (Trenimon, Bayer) or mitomycin-C, micronuclei are found in the cytoplasm of the various bone marrow cells; erythroblasts, myeloblasts, myelocytes, erythrocytes, etc. Schmid and his colleagues (*7,8*) have reported that the majority of the micronuclei detected in such preparations are found in the erythrocytes. Moreover, when treatment time with the clastogenic agent is between 24 and 30 hr, the majority of these micronuclei are found in the newly formed polychromatic erythrocytes (*8*).

For the purpose of detecting genetic damage in the form of chromosome disrupting capacity the polychromatic erythrocytes of the bone marrow have some very useful characteristics. The expulsion of the mammalian erythrocyte nucleus follows the final mitotic division by several hours, but the resulting enucleated erythrocyte retains its cytoplasmic basophilia for approximately 24 hr after nuclear expulsion. In addition, it appears that micronuclei in the cytoplasm of these cells are not expelled with the nucleus. It is therefore possible after 24 hr of treatment to examine the bone marrow of a test animal and discriminate between erythrocytes formed during the treatment time and those formed prior to exposure. In this situation each animal may be considered to serve as its own control inasmuch as the frequency of micronuclei in the polychromatic erythrocytes, formed during treatment, may be compared with the frequency of micronuclei in the normochromatic erythrocytes, formed prior to treatment. Any clastogenic response during the 24-hr time interval would result in an increase in the frequency of micronuclei only in the polychromatic erythrocyte population. Work done with two strong alkylating mutagens, Trenimon and mitocycin-C, has produced results which are in agreement with these expectations and which will be discussed at the end of this paper.

Preparation and Evaluation of Bone Marrow Smears

One of the most attractive aspects of the micronucleus test is the relative speed with which it can be done. The procedure is considerably less time consuming than either *in vivo* anaphase or metaphase analysis, both in terms of slide preparation and evaluation.

At the appropriate time after treatment, bone marrow samples are aspirated into calf serum, centrifuged, and smears made from the resuspended pellet of cells. When experimental animals are used they may be sacrificed and the shaft of the femur flushed with serum. Smears are air-dried and stained with Wright's stain or May-Grunwald-Giemsa (*8*).

Evaluation of the bone marrow preparations involves examination of from 2000 to 5000 erythrocytes per specimen, all other cell types being ignored. The total numbers of polychromatic erythrocytes with and without micronuclei as well as the total number of normochromatic erythrocytes with and without micronuclei are recorded. Interpretation is relatively straightforward, and if only the erythrocytes are examined, the evaluation requires a minimum of training and may be performed quite reliably by relatively unskilled microscopists. Several potential difficulties should however be mentioned. The first involves the discrimination of the polychromatic erythrocytes themselves. Particularly in negative control specimens or the lower treatment dose levels the polychromasy is variable and is manifest as a continuum from blueish basophilic erythrocytes to mature pinkish normochromatic erythrocytes, necessitating a decision on the majority of cells on the slide. An additional source of potential difficulty involves the presence, particularly in rat bone marrow smears, of basophilic granules from ruptured basophilic myelocytes which have staining chracteristics and morphology similar to micronuclei. These granules may be mistaken for micronuclei when they are superimposed over the erythrocytes in the area of the ruptured

myelocytes. Care must be taken to distinguish between such granules on the surface of the erythrocytes and true micronuclei present within the cytoplasm. Platelets may also be a source of confusion to the novice, but their characteristic diffuse pale staining appearance should make discrimination of superimposed platelets a matter of little real difficulty.

Effects of Strong Mutagens

Preliminary work with the micronucleus test has to date been confined to experiments with a very few potent clastogenic agents, including Trenimon and mitomycin-C. Matter and Schmid (*8*) have demonstrated a dose-dependent increase in the incidence of micronuclei in the bone marrow erythrocytes of six species of laboratory mammals by using Trenimon in doses ranging from 0.031 to 0.25 mg/kg. In another similar study, Boller and Schmid (*7*), utilizing Trenimon in the Chinese hamster, reported that the dose-dependent increase in formation of micronuclei is highly correlated with the results of cytogenetic studies on the incidence of chromosome aberrations obtained under identical experimental conditions utilizing metaphase analysis. The levels of the drug found necessary to cause an increase in detectable aberrations *in vivo* using the two tests were very similar.

The results of a pilot study done with mitocycin-C in our laboratory are found in Table I and will serve to illustrate some of the strengths and limitations of the test. In this experiment female Amsterdam rats were treated with 3μg/g body weight of mitomycin-C for 24 and 48 hr prior to sacrifice (groups 3, 4, 5, 6, Table 1). Two groups of rats (3 and 5) also received 4μg/g body weight of colcemide 4 hr prior to killing in order that bone marrow metaphase chromosome preparations could be analyzed in addition to micronuclei from the same sets of animals. One group (1) received only colcemide, and another group (2) received no treatment.

Table 1. Female Amsterdam rats 10–15 weeks of age treated with colcemide and mitomycin-C.[a]

Group[b]	Animals	Treatment	Time, hr	Micronuclei in normochromatic RBC	Micronuclei in polychromatic RBC	Micronuclei in total RBC	Polychromatic / Normochromatic	Cells with metaphase aberrations
1	3	Colcemide	4	7/4972 (0.14%)	4/1028 (0.39%)	11/6000 (0.18%)	1028/6000 (17.1%)	1/150 (0.7%)
2	3	—	—	2/4132 (0.05%)	4/1868 (0.21%)	6/6000 (0.10%)	1868/6000 (31.1%)	—
3	3	Mitomycin-C + colcemide	24 4	4/5037 (0.08%)	11/963 (1.14%)	15/6000 (0.25%)	963/6000 (16.1%)	67/150 (44.7%)
4	3	Mitomycin-C	24	3/5017 (0.06%)	10/983 (1.02%)	13/6000 (0.22%)	986/6000 (16.6%)	—
5	3	Mitomycin-C + colcemide	48 4	8/5721 (0.14%)	2/279 (0.72%)	10/6000 (0.17%)	279/6000 (4.7%)	4/150 (2.7%)
6	2	Mitomycin-C	48	19/3825 (0.50%)	6/175 (3.43%)	25/4000 (0.63%)	175/4000 (4.4%)	—

[a] Colcemide: 4μg/g, 1 injection IP; mitomycin-C: 3μg/g, 1 injection IP.

[b] Group 1: female rats treated with 4μg/g body weight of colcemide and sacrificed 4 hr later; group 2: untreated negative controls; group 3: animals received 3μg/g mitomycin-C and were sacrificed 24 hr after treatment (this group also received 4μg/g colcemide 4 hr prior to death; group 4: received 3μg/g mitomycin-C 24 hr prior to sacrifice; group 5: treated with mitomycin-C 48 hr and colcemide 4 hr prior to sacrifice; group 6: received only mitomycin-C 48 hr prior to sacrifice.

In the micronucleus test it would be expected that the incidence of micronuclei in normochromatic erythrocytes would remain constant through the first 24 hr of clastogen treatment and increase only after erythrocytes formed during the treatment interval become normochromatic (see groups 2, 4 and 6). It would also be expected that the incidence of micronuclei in the polychromatic erythrocytes would increase significantly after 24 hr of treatment (see groups 2 and 4). Both of these expectations are borne out by these data. Matter and Schmid (*8*) have suggested that the latter effect may be maximized if two treatments, rather than one, are given 24 hr apart and the animals sacrificed 6 hr after the last treatment.

Since it would be useful to be able to compare metaphase aberrations with micronuclei production in the same animals, half of the control animals and half of the two mitomycin-treated groups received an injection of colcemide 4 hr prior to sacrifice. It seems evident even from these limited data that the 4-hr colcemide treatment does tend to influence the frequency of micronuclei found in the erythrocytes (groups 1, 2, 5, and 6). Only in the 24-hr mitomycin treatment groups (groups 3 and 4) was such an effect not detected. Further work will of course be required to properly evaluate these initial observations. It should be noted however, that the colcemide-treated control rats had proportionately fewer polychromatic erythrocytes in their marrow than the untreated control group, and it is conceivable that the colcemide exerted this latter effect by blocking nuclear expulsion through disruption of microtubule formation. The decrease in the ratio of polychromatic to normochromatic erythrocytes with length of mitomycin treatment (see groups 2, 4 and 6) is construed as evidence of its toxic effect on marrow components.

For purposes of rapid screening of potential clastogenic agents it appears that the micronucleus test holds considerable promise. In our hands the time required for counting 2000 erythrocytes averaged about 15 min. This compares with approximately 1 hr for counting and analysis of 50 metaphase spreads and nearly 2 hr for analysis of 50 rat bone marrow anaphase figures.

It is suggested that a more rigorous evaluation of the micronucleus test as an *in vivo* method for mutagenicity screening must await further experimentation utilizing a variety of both strongly and weakly mutagenic compounds and sensitivity comparisons with more firmly established mutagenicity testing protocols.

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