The Development and Preliminary Use of an *In Vitro* Test for Susceptibility to Induced Chromosomal Damage

by

Joseph E. Minor, Jr.
Department of Biology

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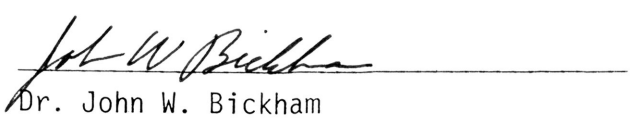

Dr. John W. Bickham

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THE DEVELOPMENT AND PRELIMINARY USE OF AN *IN VITRO* TEST FOR SUSCEPTIBILITY TO INDUCED CHROMOSOMAL DAMAGE

JOSEPH E. MINOR, JR.

Abstract

Minor Jr., Joseph E. (Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, Texas, 77843) 1983. The development and preliminary use of an *in vitro* test for susceptibility to induced chromosomal damage. Syst. Zool. 00:000-000. A new *in vitro* test for susceptibility to induced chromosomal damage is described. It involves treating primary fibroblast cultures with a known clastogen, bleomycin. The test differs from those previously published in that it makes use of fibroblast cell lines that are also used for standard cytosystematic procedures. Preliminary results from tests carried out on *Myotis lucifugus* and *Peromyscus leucopus* are discussed.

Data comparing susceptibility to induced chromosomal damage between species and between members of a species are sparce. The studies that have been done (e.g. Hsu *et al.*, 1981; Leonard, *et al.*, 1982) typically use lymphocyte cultures. Phytohemagglutinin stimulated cultures offer only one attempt at obtaining a usable karyotype, while virally transformed lymphoid lines rapidly undergo aneuploid changes. To avoid these problems, the test described here utilizes primary fibroblast cell lines routinely established in the lab for standard cytosystematic work. The test is in the form of an *in vitro* challenge test: the cells are treated with bleomycin and then harvested and checked for chromosomal aberrations. Bleomycin was chosen as the chemical clastogen (chromosomal breaking agent) since it comes closest to imitating the random chromosomal damage pattern produced by X-rays (Painter, 1982).

METHODS AND MATERIALS

Primary fibroblast cultures were established from ear biopsies of Myotis lucifugus (little brown Myotis) and Peromyscus leucopus (white-footed mouse). The cultures were maintained in Ham's F10 medium fortified with 16% fetal calf serum.

Bleomycin (Sigma) was administered as an aqueous solution based on the assumption that one unit contained approximately one milligram of bleomycin (Dr. Lorraine Cherry, personal communication). This is only an approximation since biological activity (units) and mass (milligrams) cannot be directly related due to the variability of terminal amine moieties in bleomycins (Muller and Zahn, 1977; Huff, 1982).

Two subcultures are treated with bleomycin at a final concentration of 10 µg/ml while two others are treated with distilled water (as controls). After 30 minutes, the cultures are washed once with Hanks and then are refed with normal medium. The cells are then allowed to recover for 24 hours.

Twenty-one hours into the recovery period Velban is added at a final concentration of 0.1 µg/ml. At 24 hours, the cells are washed twice with Hanks, and then the cells are removed from the flasks by trypsinization. All of the medium, Hanks, and trypsin is collected and the cells are pelleted in a clinical centrifuge. The cells are then resuspended in hypotonic (medium diluted one to four with distilled water) and incubated for 25 minutes at 37°C. The cells are then fixed in Carnoy's fixative and dropped on microscope slides flooded with water. The slides are air dried and stained in 2% Giemsa.

The slides are then coded and scored blind. Fifty metaphase

spreads are scored per treatment. The aberrations are recorded by number and type. The data are converted to number of breaks by the rules established by Hsu et al., (1977). Differences in numbers of induced aberrations between treatments were checked statistically by the Wilcoxon pair test under the null hypothesis that differences seen were due to random variation.

RESULTS

It should be noted that several treatment protocols were attempted before the one described was chosen. These all consisted of treatments of 10 μg/ml and 30 μg/ml of bleomycin under different treatment times: 5 hours, 5 hours with recovery times up to 6 hours, 4 hours with Velban added, and 1 hour with 24 hours of recovery. All of these protocols yielded too few metaphase spreads for analysis.

Using the described protocols, four individuals of two species were tested for their susceptibility to induced chromosomal damage. In addition, one individual was tested twice to check for reproducibility of results. A typical metaphase spread from a control treatment is shown in Figure 1. Figure 2 shows damage associated with bleomycin treatment of cells from the same individual. Breaks, such as those shown by the arrows, were scored and the data tabulated. The data are summarized in Table 1. All possible comparisons (bleomycin vs. control, individual vs. individual, and species vs. species) were checked for statistical differences. The following results are of importance:

1) in all cases, the bleomycin treated cells were significantly more damaged than the controls ($\alpha < 0.01$),

2) the protocol shows low variation, i.e. the two tests on the same

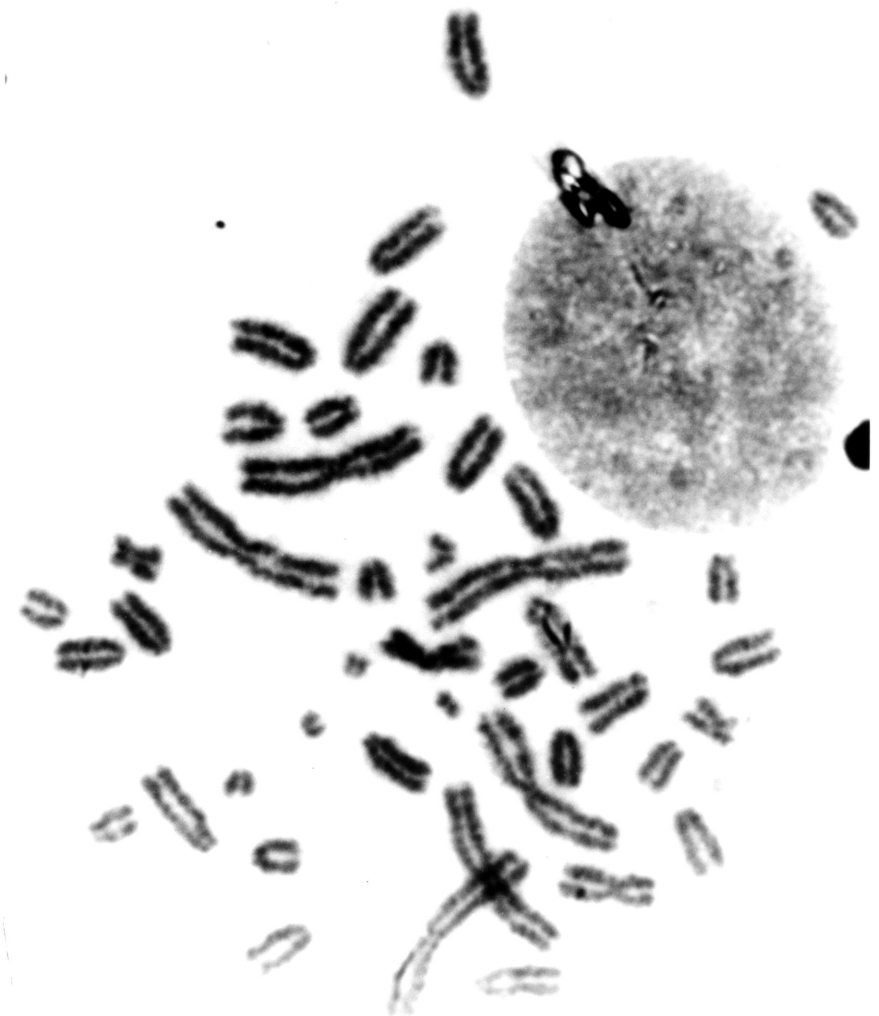

Figure 1: Metaphase spread of Myotis lucifugus chromosomes from control culture (AK4717III).

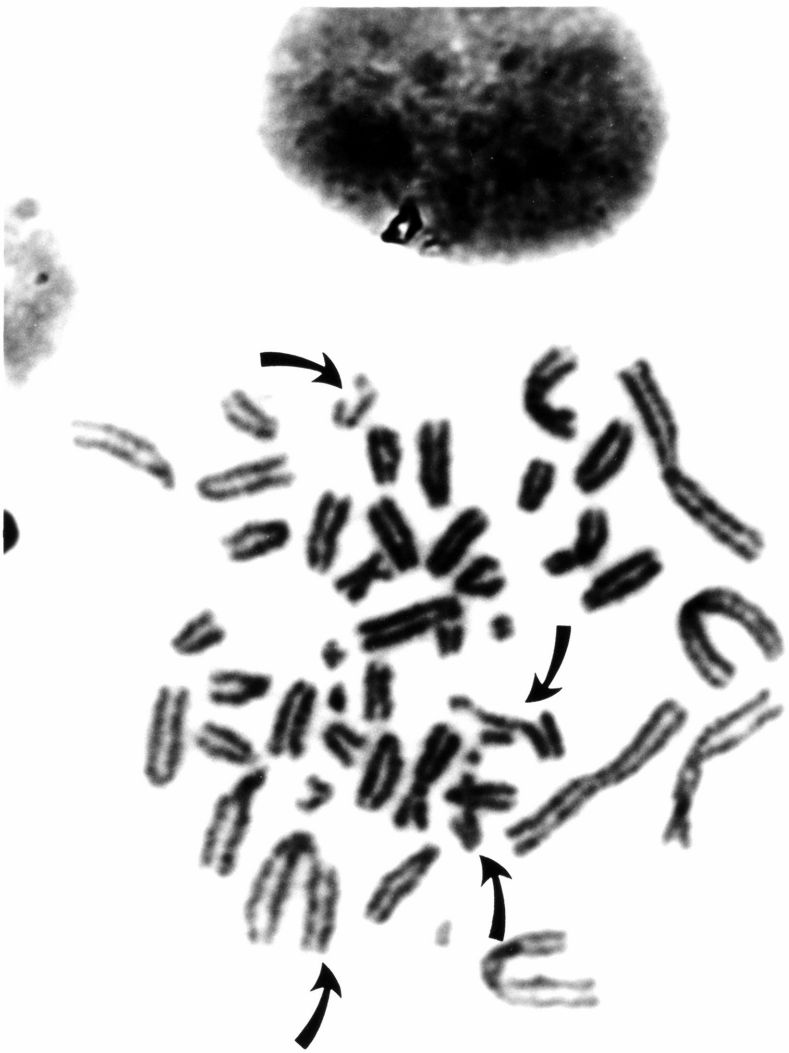

Figure 2: Metaphase spread of Myotis lucifugus chromosomes from bleomycin treated culture (AK4717III). Arrows show chromosomal aberrations.

Table 1: Summary of data collected on five individuals.

INDIVIDUAL		CONTROL		BLEOMYCIN	
		% cells damaged	# lesions/cell	% cells damaged	# lesions/cell
AK4717III	(*Myotis* #1)	12	0.18	44	0.66
AK4717IV	(*Myotis* #1)	10	0.12	44	0.74
AK4718I	(*Myotis* #2)	8	0.08	32	0.68
AK2498I	(*Peromyscus* #1)	6	0.12	42	1.22
AK7501I	(*Peromyscus* #2)	0	0	14	0.46

individual gave very similar results ($\alpha >> 0.9$), and

3) the only other statistically significant difference was between the two Peromyscus individuals after bleomycin treatment ($\alpha < 0.05$).

DISCUSSION

While bleomycin causes random breakage of DNA at concentrations of 12 mg/ml (Muller and Zahn, 1977), at the concentration used for these experiments (10 µg/ml) bleomycin has been shown to act at specific sites both at the DNA (Muller and Zahn, 1977; Loyd et al., 1978) and chromatin (Kuo and Hsu, 1978a and 1978b) levels. This must be kept in mind in interpreting data, particularly when a given species contains AT rich DNA (because of thymine specificity, see Muller and Zahn, 1977). Despite these problems, bleomycin is probably the best chemical agent at imitating the random physical breakage pattern produced by X-rays (Painter, 1982). The use of a chemical agent is desirable since dosage is easier to monitor and X-ray equipment is not available to our lab.

The protocol described dependably yields high quantities of metaphase spreads for analysis. Fibroblast cultures are more readily manipulated with respect to medium changes than lymphocytes and can be subcultured readily for multiple usage in the lab. On the negative side, fibroblasts are more sensitive to bleomycin than lymphocytes (probably due to underproduction of BLM hydrolase, see Yoshioka et al., 1978) so the necessary recovery period may give the cells time to repair induced damage (Sognier et al., 1982; Hsu et al., 1982). However, the species diversity obtainable by using cultures established for standard lab work should outweigh these disadvantages.

The data generated from the use of this test are, and will continue

to be, useful in understanding chromosomal evolution. For a chromosomal change to become incorporated between two groups of organisms, a change must occur, then become fixed in one group relative to the other. Current models of chromosomal evolution (Wilson et al., 1975, 1977; Bush et al., 1977; Bickham and Baker, 1979; Marayama and Imai, 1981) either implicitly or explicitly assume occurence rates are constant and differences in rates of chromosomal evolution are soley due to different rates of fixation. This assumption has not been tested well. In fact, data from intraspecies comparisons (Homo sapiens: Hsu et al., 1981; Schroeder, 1982; Mus musculus: Halpern et al., 1972; Emerit et al., 1974, 1980) and preliminary interspecies data (Leonard, et al., 1982) suggest this assumption may be incorrect. One way to further test the constant occurence assumption is by in vitro challenge tests such as the one described here. While there are theoretical objections to such challenge tests (i.e. -- mutations induced in vitro may not accurately reflect mutations occurring naturally in meiotic tissue), challenge tests represent a practical way of achieving quantities of data amenable to statistical tests.

The two species chosen for the initial tests (Myotis lucifugus and Peromyscus leucopus) are particularly well suited for comparison in the bleomycin challenge test. Myotis lucifugus is a very karyotypically conservative species, with no chromosomal polymorphism within the species, only one chromosomal change within the genus, and since the Myotis karyotype is primitive for the family Vespertilionidae, only one chromosomal change in the Myotis lineage since the family was founded (Baker and Bickham, 1980). Peromyscus leucopus, on the other hand, exhibits rapid chromosomal evolution. It shows species polymorphism (Baker et al.,

manuscript in preparation) and numerous changes within the genus Peromyscus and the family Cricetidae (Robbins and Baker, 1981). Briefly, Myotis and Peromyscus come from the extreme ends in the spectrum of rates of chromosomal evolution in mammals. Therefore, if one were to expect a difference in the amount of induced chromosomal damage, it should be seen between these two species. However, based on the data reported here, there were no statistically significant differences between the two species. This supports the constant occurence rate assumption of the chromosomal evolution models. Another inference which can be made is that statistically significant difference between the two Peromyscus individuals may reflect the karyotypic diversity within the Peromyscus lineage. Clearly, more data are needed before full confidence can be expressed in either conclusion.

What is more important is that an effective in vitro challenge test using fibroblasts has been developed. It has been shown that the drug is having the desired effect and that the results are highly reproducible. Therefore, the bleomycin in vitro challenge test is ready for use on any fibroblast cell line established in the lab. This should allow analysis of many species for susceptibility to induced chromosomal change, thus expanding our understanding of chromosomal evolution.

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