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INTER-LABORATORY CALIBRATION PROGRAM FOR THE MOUSE MICRONUCLEUS TEST

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ABSTRACT

The micronucleus test on mammalian bone marrow is widely accepted as a "screening" test for chromosome anomalies induced by clastogenic agents. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) in the bone marrow of mice treated with cyclophosphamide (CP), bleomycin (BLEO) and benzidine (BZN) was determined in three different laboratories. Although the experiments were conducted independently, the results were quite similar for all three laboratories. The mean MNPCE frequencies induced by CP and BLEO were not statistically different. Only for the group treated with a single BZN dose were the mean frequencies statistically different between laboratories two and three. The homogeneous results obtained by the three laboratories for the micronucleus test indicate the efficacy of the method and its applicability for the identification of clastogenic agents.

INTRODUCTION

The *in vivo* micronucleus test developed by Schmid (1975, 1976) is an alternative for cytogenetic studies on mammalian bone marrow. It provides a direct measurement of the frequency of structural or numerical chromosome aberrations (Mavournin *et al.*, 1990). Salamone *et al.* (1980), Heddle *et al.* (1983) and Sarto *et al.* (1987) have emphasized the greater rapidity and ease in preparation and analysis of slides

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with this method when compared with tests based on the detection of chromosome aberrations and sister chromatid exchanges.

According to Sarto *et al.* (1987), the micronucleus originate from chromosome regions which lag or migrate irregularly during anaphase. These regions may be produced by chromosome breaks or by intact chromatids which lag behind due to disturbances in the mitotic spindle. During telophase, these chromosome regions may be included in daughter cells and fuse with the main nucleus or form one or more smaller secondary nuclei called micronuclei. Thus, micronuclei are formed only during cell division and can be observed, for example, in bone marrow erythrocytes.

Young erythrocytes formed by expulsion of the nuclei from erythroblasts present a bluish color when stained with Giemsa because they still contain ribosomal RNA in the cytoplasm, and they are called polychromatic erythrocytes (PCEs). Mature erythrocytes present an orange color when stained with Giemsa and are called normochromatic erythrocytes (NCEs). According to Heddle *et al.* (1983), the distinction between PCE and NCE is of fundamental importance to confirm whether the increased incidence of micronuclei is actually due to exposure to a given chemical agent, since there is a constant migration of normochromatic cells from the peripheral circulation to bone marrow.

According to Mavournin *et al.* (1990), the expulsion of the nucleus in mouse bone marrow occurs 6 h after the last mitosis and the PCE stage lasts 12 to 24 h in bone marrow and a similar time in peripheral blood before the cells are transformed into NCEs. Thus, no micronucleus induced by a chemical agent would be detected before 6 h after treatment.

Several investigators have examined the protocol of the micronucleus test in order to develop an adequate treatment schedule and to determine the time till the occurrence of a maximum frequency of a micronucleated PCEs. According to Adler and Kliesch (1990), the time till maximum response may differ according to the chemical agent used or the administration route. Mavournin *et al.* (1990) consider the variation in micronucleus frequency with time to be a complex function involving many factors, so that the exact time of maximum micronucleated PCE occurrence cannot be predicted. In many cases, the peak will occur between 24 and 60 h after treatment. Yamamoto and Kikuchi (1981), Salamone *et al.* (1980), Salamone and Heddle (1983), Preston *et al.* (1987) and Mavournin *et al.* (1990) have proposed different schemes for the treatment schedule and no general consensus has been reached to date. According to Mac Gregor *et al.* (1987), there is not yet sufficient evidence to support the exclusive use of a specific treatment schedule for all substances tested.

Little has been done to elaborate a viable procedure for the analysis of data obtained by the micronucleus test (Amphlett and Delow, 1984). Mackey and Mac Gregor (1979) have proposed a negative binomial distribution for the incidence of micronucleated erythrocytes. However, according to Hart and Engberg-Pedersen (1983), this method is not appropriate for this type of estimate. The latter investigators stated that their results followed a binomial distribution and that treated and control groups could be compared by the chi-square test. Amphlett and Delow (1984) have proposed that the data for the incidence of micronucleated erythrocytes fit a Poisson distribution.

In view of the lack of a defined protocol and of the growing importance of the micronucleus test among cytogenetic tests, the 13 Brazilian mutagenesis laboratories which participate in the Integrated Mutagenesis Program (PIMUT) met several times to set up a calibration of the test. The three laboratories selected for the present project were the Laboratório de Genética Toxicológica da Faculdade de Medicina Veterinária, Federal University of Bahia (laboratory 1); Laboratório de Citogenética e Mutagênese da Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, University of São Paulo (laboratory 2); and the Departamento de Genética, Federal University of Rio Grande do Sul (laboratory 3).

The project consisted of a determination of the frequency of micronuclei in mouse bone marrow induced by the genotoxic drugs cyclophosphamide (CP), bleomycin (BLEO) and benzidine (BZN) obtained from a single fixed source. The project started in 1990 and was sponsored by the National Council for the Advancement of Science (CNPq) and by the Agency for the Financing of Studies and Projects (FINEP). The same substances were tested separately by the three Brazilian laboratories using the same treatment schedule.

MATERIAL AND METHODS

Animals

Male Swiss mice aged approximately three weeks were donated to the three laboratories by the Butantan Institute, São Paulo. They were treated at eight weeks of age. The animals received commercial ration and water during the acclimation period and during the experiment.

Chemical agents

The chemical agents used were cyclophosphamide (CP) - Enduxan-Pravaz, bleomycin (BLEO) - Sigma, and benzidine (BZN) - Blenoxane Bristol, supplied to the three laboratories by the Integrated Mutagenesis Program, Rio de Janeiro.

Doses and times for sample collection

During the first phase of the experiment, CP and BLEO were used in a single bolus dose of 50 mg/kg and 80 mg/kg, respectively. Thirty animals divided into three groups of 10 were used. One group was treated with CP, another with BLEO and the third group (control) was divided into two subgroups, one for each drug. Each animal in the treated groups was injected intraperitoneally with the chemical substances, dissolved in 0.3 ml distilled water. Control animals received the same volume of distilled water.

During the second phase of the experiment, BZN was applied at a dose of 100 mg/kg. Three groups of 10 animals each were used and treated according to the schedule presented in Table I. Each animal was injected intraperitoneally with the drug dissolved in 0.3 ml corn oil. The control group consisted of five animals, which received a single intraperitoneal injection of corn oil.

Table I - Scheme of intraperitoneal treatment (#) with 100 mg/kg b.w. of benzidine and the sacrifice of mice (O).

Groups	Time hours			
	0	24	48	72
1	#	O		
2	#	#	O	
3	#	#	#	O

Micronucleus test

The micronucleus test was performed according to the guidelines of Mac Gregor *et al.* (1987). The animals were killed by cervical dislocation 24 h after treatment, both femurs were removed and cleaned, and the epiphyses were cut to expose the medullary canal. The needle of a syringe containing 1 ml fetal calf serum was introduced into the canal, and the femur was placed in a centrifuge tube containing 2 ml fetal calf serum, resting against the tube wall to prevent escape from the syringe. The bone marrow was aspirated and resuspended several times until it spread through the serum as a fine suspension. The material was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed with a Pasteur pipette, the sediment carefully aspirated with the pipette, and one drop placed on a clean slide. A fine smear was obtained with the aid of a coverslip. After 24 hours, the material was fixed in absolute methanol for 10 minutes. On the following day, the material was stained with Giemsa diluted 1:10 in sodium phosphate buffer, pH 6.8.

Analysis of the slides

Cells were analyzed under a light microscope with a 100X immersion objective. The number of micronucleated cells was counted in 2000 PCEs per animal. All the slides were analyzed in a blind test. During analysis, artifacts were excluded from the counts according to the criterion of Schmid (1976) whereby artifacts are refractory when focused.

Statistical analysis

The statistical differences between control and treated groups were determined by the chi-square test (Pereira, 1991). The results obtained by the three laboratories were compared by analysis of variance and by the Student-Newman-Keuls test.

RESULTS

The frequencies of micronucleated PCEs induced by CP, BLEO and BZN obtained by the three laboratories are presented in Table II. Although the experiments were carried out independently, the results were quite similar. All treatments produced significantly more micronucleated PCEs than the respective controls ($P < 0.01$).

Table II - Frequencies (mean ± SD) of micronucleated polychromatic erythrocytes in % induced by cyclosphosphamide (CP), bleomycin (BLEO) and benzidine (BZN) detected by laboratories 1, 2 and 3 (see text).

Treatment	Dose (mg/kg)	Time after injection (h)	Lab. 1	Lab. 2	Lab. 3
Water	-	24	0.20 ± 0.10	0.20 ± 0.09	0.18 ± 0.05
CP	50	24	2.89 ± 1.14	2.86 ± 0.53	3.13 ± 0.53
Water	-	24	0.18 ± 0.02	0.20 ± 0.08	0.13 ± 0.04
BLEO	80	24	1.05 ± 0.35	0.91 ± 0.16	1.10 ± 0.23
Oil	-	24	0.19 ± 0.04	0.29 ± 0.06	0.20 ± 0.04
BZN	1 x 100	24	0.59 ± 0.16	0.49 ± 0.16	0.65 ± 0.08
	2 x 100	48	0.97 ± 0.29	0.92 ± 0.33	0.98 ± 0.33
	3 x 100	72	0.79 ± 0.22	0.76 ± 0.19	0.88 ± 0.20

Mean micronucleated PCE frequencies induced by CP and BLEO did not differ significantly (analysis of variance). However, for the group treated with a single BZN dose (100 mg/kg), the mean frequencies of micronucleated PCEs differed between laboratories two and three ($P < 0.05$).

DISCUSSION

The micronucleus test was first recommended in Brazil in 1988 by the Brazilian Environmental Institute (IBAMA) as part of a battery of tests to be used for the genotoxic evaluation of some chemical compounds. The Integrated Mutagenesis Program coordinated the intercalibration of the micronucleus test in order to standardize the conditions to be used by the three laboratories which participated in this project.

Cyclophosphamide yields clearly positive results in the micronucleus test (Tinwell *et al.*, 1990) and therefore is used as a positive control by several investigators (Adler and Kliesh, 1990; Cihak and Vontorkova, 1990; George *et al.*, 1990). The results of our bone marrow experiments using CP were also positive, in agreement with these investigators.

Our results with bleomycin (BLEO) differed from those obtained by Maier and Schmid (1976), who did not observe a significant increase in micronucleated PCEs. However we used 80 mg/kg, double that used by these investigators.

When Tice *et al.* (1990) used a single treatment with benzidine (BZN) there was no significant increase in micronucleated PCEs, but a significant increase was obtained after the use of two or three treatments. We found a significant increase for all treatment groups, including those submitted to a single treatment. In this last group, however, the frequency of micronucleated PCEs was the lowest compared to the other treatments in all three participating laboratories.

No significant difference in mean frequency of micronucleated PCEs induced by CP, BLEO and BZN (multiple treatment) was observed among the results obtained by the three laboratories. A significant difference in micronucleated PCE frequency was observed between laboratories two and three when a single BZN dose was used, though among 18 treatments, one difference is expected by chance at $\alpha = 5\%$. Taken as whole, the results of the micronucleus test were similar in all three laboratories, supporting the application and utilization of this method for the identification of genotoxic agents.

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RESUMO

O teste de micronúcleo em medula óssea de mamíferos é bem aceito como teste de "triagem" para anomalias cromossômicas induzidas por agentes clastogênicos. A análise da freqüência de eritrócitos policromáticos micronucleados (MNPCEs) de medula óssea de camundongo tratado com ciclofosfamida (CPA), bleomicina (BLEO) e benzidina (BZN) foi realizada em três laboratórios. Embora os experimentos tenham sido conduzidos independentemente, os resultados foram muito semelhantes para os três laboratórios. As freqüências médias de MNPCEs induzidas por CPA e BLEO não foram estatisticamente diferentes; somente para o grupo tratado com uma única dose de benzidina as freqüências médias foram estatisticamente diferentes entre os laboratórios 2 e 3. A homogeinidade obtida entre o teste de micronúcleo dos três laboratórios indica a eficiência do método e a sua aplicabilidade na identificação de agentes clastogênicos.

REFERENCES

Adler, I.D. and Kliesh, U. (1990). Comparison of single and multiple treatment regimes in the mouse bone marrow micronucleus assay for hidroquinone (HQ) and ciclophosphamide (CP). *Mutation Res. 234*: 115-123.

Amphlett, G.E. and Delow, G.F. (1984). Statistical analysis of the micronucleus test. *Mutation Res. 128*: 161-166.

Cihak, R. and Vontorkova, M. (1990). Activity of acrylamide in single, double, and triple dose mouse bone marrow micronucleus assays. *Mutation Res. 234*: 125-127.

George, E., Wootton, A.K. and Gatehouse, D.G. (1990). Micronucleus induction by azobenzene and 1,2 dibromo 3 chloropropane in the rat: Evaluation of a triple dose protocol. *Mutation Res. 234*: 129-134.

Hart, J.W. and Engberg-Pedersen, H. (1983). Statistics of the mouse bone-marrow micronucleus test: counting, distribution and evaluation of results. *Mutation Res. 111*: 195-207.

Heddle, J.A., Hite, M., Kirkhart, B., Mavournin, K., Mac Gregor, J.T., Newell, G.W. and Salamone, M.F. (1983). The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene, Tox. Program. *Mutation Res. 123*: 61-118.

Mac Gregor, J.T., Heddle, J.A., Hite, M., Margolin, B.H., Ramel, C., Salamone, M.F., Tice, R.R. and Wild, D. (1987). Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. *Mutation Res. 189*: 103-112.

Mackey, D.E. and Mac Gregor, J.T. (1979). The micronucleus test: Statistical design and analysis. *Mutation Res. 64*: 195-204.

Maier, P. and Schmid, W. (1976). Ten model mutagens evaluated by micronucleus test. *Mutation Res. 40*: 325-338.

Mavournin, K.H., Blabuy, D.M., Cimino, M.C., Salamone, M.F. and Heddle, J.A. (1990). The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene. Tox. Program. *Mutation Res. 239*: 29-80.

Pereira, C.A.B. (1991). Testes estatísticos para comparar proporções em problemas de citogenética. In: *Mutagênese, Carcinogênese e Teratogênese: Métodos e Critérios de Avaliação* (Rabello-Gay, M.N., La Regina-Rodrigues, M.A. e Monteletone Neto, R., eds.). Rev. Brasil. Genet., Ribeirão Preto, SP, pp. 83.

Preston, R.B., Dean, D.J., Galloway, S., Holden, H., McFee, A. and Shelby, M. (1987). Analysis of chromosome aberrations in bone marrow cells. *Mutation Res. 189*: 157-165.

Salamone, M.F. and Heddle, J.A. (1983). The bone marrow micronucleus assay: Rationale for a Revised Protocol. In: *Chemical Mutagens* (Serres, F.J., ed.). Plenum Press, New York, vol. 8, pp. 111-149.

Salamone, M., Heddle, J., Stuart, E. and Katz, M. (1980). Studies on 3 Model Agents, mitomycin C, cyclophosphamide and Dimethylbezanthracene. *Mutation Res. 74*: 347-356.

Sarto, F., Finotto, S., Giacomelli, L., Mazotti, D., Tomanin, R. and Levis, A.G. (1987). The micronucleus assay in exfoliated cells of the human buccal mucosa. *Mutagenesis 2*: 11-17.

Schmid, W. (1975). The micronucleus test. *Mutation Res. 31*: 9-15.

Schmid, W. (1976). The micronucleus test for cytogenetic analysis. In: *Chemical Mutagens* (Hollender, A., ed.). Plenum Press, New York, vol. 4, pp. 31-53.

Tice, R.R., Erexson, G.L. and Shelby, M.D. (1990). The induction of micronucleated polychromatic erythrocytes in mice using single and multiple treatments. *Mutation Res. 234*: 187-193.

Tinwell, H., Bandara, L. and Ashby, J. (1990). Activity of DMBA, DMH and CP in triple and single dose rodent bone marrow micronucleus assay. *Mutation Res. 234*: 195-198.

Yamamoto, K.I. and Kikuchi, Y. (1981). Studies on micronuclei time response and the effects of multiple treatments of mutagens on induction of micronuclei. *Mutation Res. 20*: 163-173.

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