Mutation Research, 147 (1985) 29–36
Elsevier

MTR 08568

Measurement of micronuclei in lymphocytes

Michael Fenech and Alexander A. Morley

Department of Haematology, Flinders University and Medical Cenrre. Bedford Park, South Australia 5042 (Australia)

(Received 7 August 1984)
(Revision received 11 October 1984)
(Accepted 5 November 1984)

Summary

The micronucleus technique has been proposed as a method for measurement of chromosomal damage in mitogen-stimulated human lymphocytes. Micronuclei require one cell division to be expressed and, consequently, the conventional micronucleus technique is very imprecise since the cells which have undergone only one division, and the micronuclei in them, cannot be identified separately from the total population of lymphocytes. To overcome this problem, two methods were developed to identify cells which have undergone their first mitosis.

Using an autoradiographic technique, lymphocytes were pulse-labelled with [^{3}H]thymidine at 48 h of culture, allowed to proceed through mitosis, identified by autoradiography between 72 and 84 h and micronuclei were scored in them. It was not possible to select a concentration of radiolabel which did not itself produce micronuclei and consequently the method was of no value for measuring pre-existing chromosomal damage present in vivo. However, it was capable of quantitating micronuclei produced by irradiation of lymphocytes in vitro.

In the second method, cytokinesis was blocked using cytochalasin B. Micronuclei were scored in cytokinesis-blocked cells. These were easily recognisable owing to their binucleate appearance and a large number could be accumulated by adding 3.0 μg/ml cytochalasin B at 44 h and scoring at 72 h. Cytochalasin B did not itself produce micronuclei. The cytokinesis-block method was simple to perform; the 'in vivo' micronucleus frequency in normal individuals was 4.4 ± 2.6 micronuclei/500 cytokinesis-blocked cells; and for lymphocytes irradiated in vitro there was a linear relationship between dose of radiation and number of induced micronuclei.

The cytokinesis-block method appears to be the procedure of choice for quantitating micronuclei in lymphocytes.

Micronuclei enclose acentric chromosome fragments or whole chromosomes that have not been incorporated in the main nuclei at cell division, and enumeration of micronuclei in mitogen-stimulated lymphocytes provides a simpler and statistically more precise method than karyotypic analysis for quantitation of chromosomal damage. However, as applied to human lymphocytes and other proliferating cell populations, the method has one serious disadvantage — it is necessary for a cell to undergo a mitosis in order for a micronucleus to be expressed. Thus in enumerating micronuclei in lymphocytes the number of micronuclei scored in a given number of lymphocytes depends on (a) the proportion of cells that have responded to the mitogen, (b) the proportion of the responding cells that have divided, and (c) the fate of micronuclei in cells which have divided

0165-1161/85/$03.30 © 1985 Elsevier Science Publishers B.V. (Biomedical Division)

more than once. These factors may vary greatly. both between different individuals and. depending on technical factors, within one individual; as a consequence the micronucleus method as conventionally performed on human lymphocytes is very imprecise.

In this paper we describe two approaches we have made to score micronuclei in lymphocytes which have divided only once.

The principle of the *autoradiographic method* is to pulse-label phytohaemagglutinin (PHA)-stimulated lymphocytes with [^{3}H]thymidine at 48 h. 'This allows the study of micronucleus expression in an easily recognisable proliferating sub-population that is in synchrony at S-phase. If samples are taken periodically after the pulse and autoradiography performed on the cells. it is possible to score micronuclei in labelled cells which have undergone only one division (Fig. 1). Micronuclei will not appear in labelled cells until those that were in late S at the time of pulsing progress through G_2 + M. and the level of micronuclei will continue to increase for the duration of S until a plateau is reached. The plateau will persist until cells start to go through their second division, at which time the proportion of micronuclei in labelled cells will be expected to decline, since the second division will increase the number of labelled cells without necessarily increasing the number of micronuclei. At the plateau level all of the labelled cells will have divided once and it is therefore possible to estimate the level of micronuclei at this time.

Fig. 1. A photomicrograph of a labelled cell containing a micronucleus (M). Magnification × 1000.

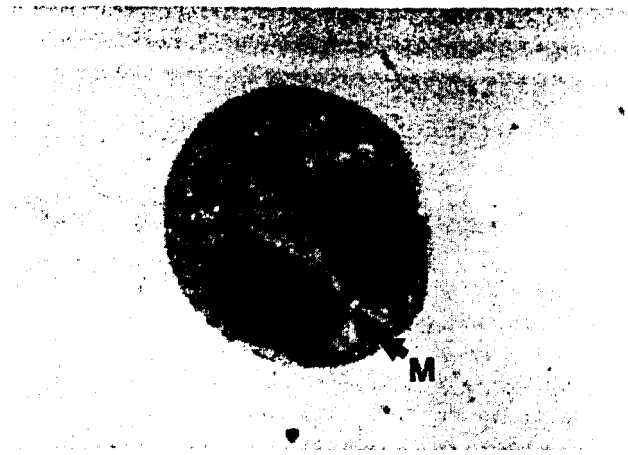

Fig. 2. A photomicrograph of a cytokinesis-blocked (CB) cell, containing a micronucleus (M). Magnification × 1000.

In the *cytokinesis-block method* micronuclei are scored only in cells that have been inhibited from undergoing cytokinesis (Fig. 2). Cytokinesis-blocked (CB) cells are easily recognisable by their binucleate appearance and they must be dividing cells which have completed nuclear but not cytoplasmic division. CB cells in lymphocyte cultures are obtained by adding cytochalasin B (Cyt-B) at 44 h so that cytokinesis is inhibited and binucleate cells are accumulated in their first division cycle.

Materials and methods

Cell culture

Blood was obtained from healthy donors aged between 20 years and 35 years. Peripheral blood lymphocytes were separated from whole blood on Ficoll-Hypaque gradients, washed twice in Hank's balanced salt solution and resuspended in McCoy's modified medium 5A containing 15% heat-inactivated foetal calf serum. The lymphocytes were cultured in 0.2-ml microwells at a concentration of 0.5×10^6 cells/ml. An optimum concentration of PHA (5 μg/ml, Burroughs Wellcome reagent-grade) was used to stimulate the lymphocytes to transform and divide in culture. The cells were cultured at 37°C in a humidified atmosphere containing 10% CO_2.

X-Irradiation

Cells were exposed to X-rays at G_0 before addition of PHA. X-Rays were delivered by a Philips RT100 at a rate of 400 rad/min at 6 mA, 100 kV and 1.7-mm Al filtration. The dose rate was measured using a Siemens dosimeter.

Autoradiographic method

Unless otherwise stated, lymphocytes were pulse labelled for 30 min with 2 μCi/ml tritiated thymidine (Radiochemical Centre, Amersham, England, spec. act. 5 Ci/mmole) at **48** h after PHA stimulation. Pulsing with [^{3}H]thymidine earlier than 48 h did not enable sufficient cells to be labelled and scored. The cells were then washed 3 times with medium at 37°C containing 10^{-5} M unlabelled thymidine and then cultured at 37°C in medium supplemented with conditioned medium as a source of interleukin-2. Conditioned medium was prepared from tonsillar lymphocytes which had been irradiated with 2000 rad X-rays, stimulated with PHA and cultured for 2 days.

For the determination of the baseline and induced level of micronuclei in labelled cells, samples from cultures were periodically removed between 52 and 96 h and two cytocentrifuge preparations of the cells on clean slides were made for each sample. The cells were allowed to dry on the slides and then fixed for 15 min in absolute methanol. Autoradiography was performed on the slides using Ilford K2 emulsion and an exposure time of one week. The emulsions were developed with Kodak D-19 developer and fixed in 30% sodium thiosulphate solution. The slides were then dried and stained with May–Grunwald–Giemsa. Both the number of micronuclei in a minimum of 1000 labelled lymphocytes and the labelled mitosis index based on 100 cells were scored.

Cytokinesis-block method

Cytochalasin B (Cyt-B, Sigma) was made up as a stock solution in dimethyl sulphoxide (DMSO) at a concentration of 2 mg/ml, divided in small portions and stored at −70°C. The stock solution of Cyt-B was thawed, diluted in saline and added 44 h after the commencement of the culture at a concentration of 3.0 μg/ml unless otherwise stated, The cultures were stopped at 72 h and two cytocentrifuge preparations of cells on slides were made per sample.

The number of CB cells varied from individual to individual with frequencies generally varying between 5 and 50%. Occasionally, trinucleate and quadrinucleate CB cells were observed but the frequency of these in cultures from 9 individuals was not more than 0.3% of the total number of multinucleate cells. Scoring of micronuclei was limited to binucleate CB cells only. Micronuclei in a minimum of 800 CB cells were usually scored but in many instances more than 1000 CB cells were scored depending on the frequency of CB cells.

Scoring of micronuclei

Slides were scored at 1000× magnification. For the identification of micronuclei published criteria were applied (Countryman and Heddle, 1976; Heddle et al.. 1978). Identification of CB cells in cell groups required careful visual examination of the individual cell boundaries of cytoplasm.

Results

Autoradiographic method

Preliminary experiments with pulse-labelled cells in culture indicated that the presence of [^{3}H]thymidine could itself induce micronuclei, presumably due to the radiation emitted with the decay of tritium. The results depicted in Fig. 3 show a direct relationship between pulsing time and the level of micronuclei in all cells (labelled

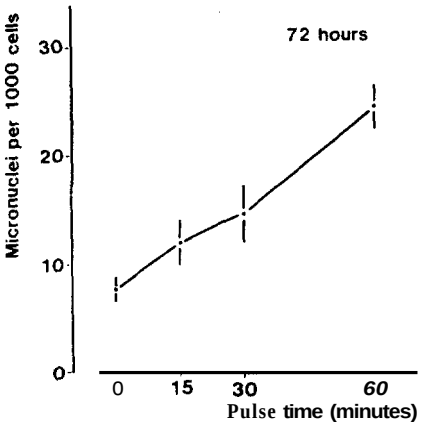

Fig. 3. The effect of pulse-labelling time on micronucleus frequency in all, i.e. labelled and unlabelled cells at **72** h. Cells were pulsed **with 2** μCi/ml tritiated thymidine. Bars indicate the standard error of the mean for cultures from 6 different individuals.

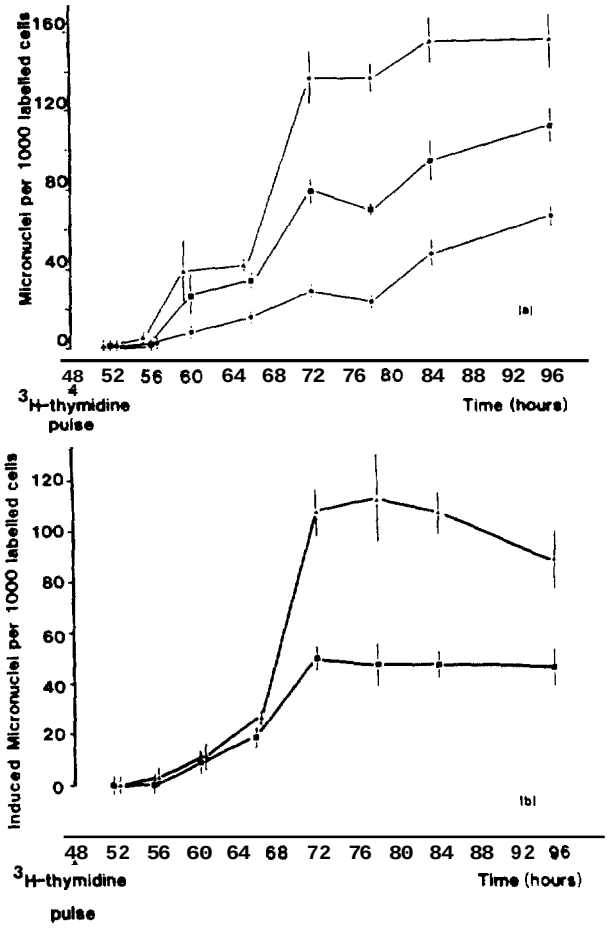

Fig. 4. Relationship between micronucleus frequency in labelled cells and time of culture. (a) Total micronuclei, (b) micronuclei induced by radiation as calculated by subtraction of the value for unirradiated cells from the total value for irradiated cells. 4 individuals were studied and the results are expressed as the mean ± 1 SE. Solid circles, unirradiated cultures; solid squares. cultures exposed to 100 rad; solid triangles, cultures exposed to 200 rad.

and unlabelled) in cultures harvested at 72 h after PHA stimulation. A pulsing time of 30 min was selected in order to provide a pulsing time that would not drastically elevate the induction of micronuclei whilst at the same time allow a high enough signal for a rapid and unequivocal detection of labelled cells by using autoradiography.

The effect of X-irradiation on production of micronuclei in labelled cells was studied in cultures from four individuals and the results are shown in Fig. 4 (a). For the unirradiated cells the number of micronuclei did not start to increase until about 56 h and there was then a progressive increase until 96 h with no sign of development of a plateau. The total number of micronuclei in the irradiated cells likewise showed no evidence of development of a plateau (Fig. 4a) but a plateau became clearly apparent when the results for the unirradiated cells were subtracted from the total in order to provide the number of micronuclei induced by the irradiation (Fig. **4b).** The plateau region which lay between approximately 72 and 84 h, gave the number of micronuclei induced by irradiation in cells that had divided only once and Fig. 7 shows the X-ray dose–response curve obtained by averaging, for each dose of X-ray. the results at 72, 78 and 84 h.

From the data shown in Fig. 4 it is also possible to obtain information on cell cycle parameters. The $G_2 + M$ phase was about 6–8 h long and S phase was estimated to be about 12–16 h long. These estimations were similar to the measurements made from the corresponding percentage labelled mitosis curve.

Cytokinesis-block method

A preliminary investigation was done to determine the optimum concentration of Cyt-B for accumulating CB cells. Lymphocytes were exposed to varying concentrations of Cyt-B, harvested and the number of CB cells per 1000 cells was scored. The results for two normal individuals are shown in Fig. 5. The optimum Cyt-B concentration appeared to be 3.0 μg/ml and this concentration was used throughout the experiments.

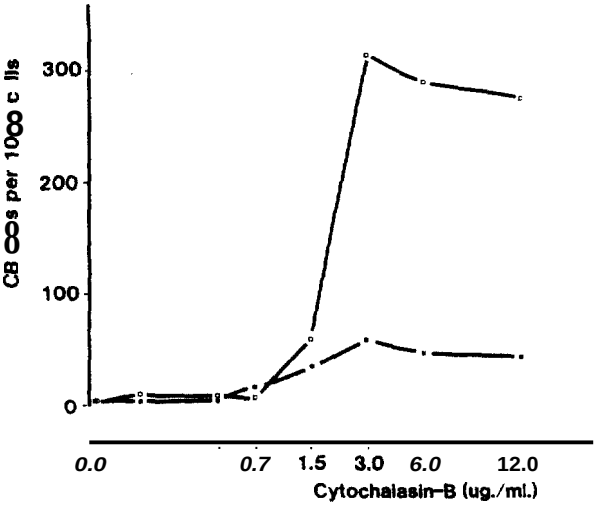

Fig. 5. The relationship between the frequency of accumulated CB cells and cytochalasin B concentration. The results for two individuals are shown. Cytochalasin B concentration is plotted on a log-scale.

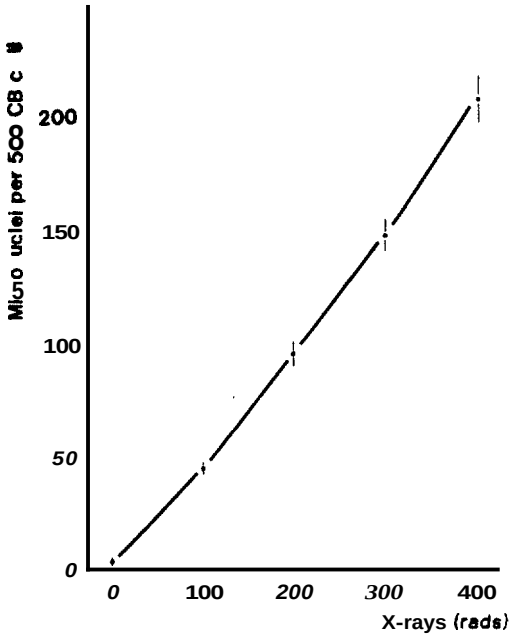

Fig. 6. The dose–response for frequency of micronuclei in CB cells in cultures exposed to X-rays. The bars indicate the standard error of the mean for cultures from 8 individuals (0, 100 and **200** rad) and two individuals (300 and 400 rad).

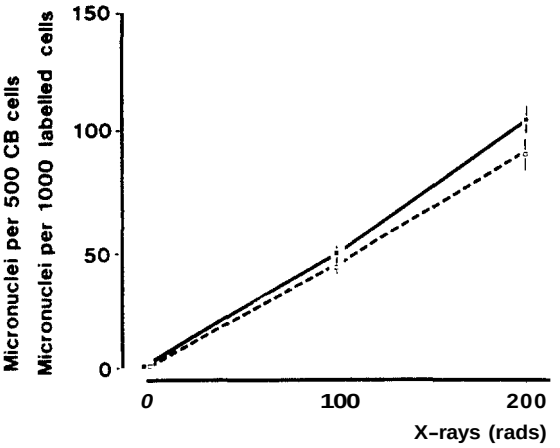

Fig. 7. A comparison of the dose–response obtained by the autoradiographic method and the CB method, for micronucleus induction after exposure to X-rays. Solid squares, results for the autoradiographic method; bars indicate the standard error of the mean for cultures from 4 individuals. Open squares, results for the CB method; bars indicate the standard error of the mean for cultures from 8 different individuals.

For **8** subjects micronuclei were enumerated in CB cells both in unirradiated and in irradiated lymphocytes. The results are summarised in Table 1 and shown in Fig. 6. The baseline number of micronuclei/500 CB cells in unirradiated lymphoeytes was very low being **4.4**± 2.6 (mean ± 1SD). There was an approximately linear and significant ($r^2 = 0.95$, p 0.01) relationship between the frequency of induced micronuclei and dose of irradiation and when analysed by linear least squares regression the line of best fit was:

$$y = 0.51D - 0.11$$

where y = number of micronuclei/500 CB cells and D = X-ray dose in rad. However, these data could be fitted equally well by the method of least squares to a power curve of. the form $y = kDn$ where $n = 1.09$, $k = 0.29$ and $r^2 = 0.94$.

In order to determine whether Cyt-B itself in-

TABLE 1

LEVELS OF MICRONUCLEI IN CYTOKINESIS-BLOCKED (CB) CELLS IN RELATION TO HIGH-LEVEL **X-RAY** DOSE

Subject	Number of micronuclei/500 CB cells				
	0 rad	100 rad	200 rad	300 rad	400 rad
JJ	7.3(2000)	46.6(2157)	98.7(1500)	157.0(1210)	222.9(1608)
MF	7.9 (833)	48.2 (840)	77.7 (850)	138.7 (898)	195.0(1092)
PT	0.9 (887)	42.7 (874)	105.9 (708)		
RE	1.7 (871)	50.8 (846)	94.9 (790)		
CG	2.9 (851)	52.0 (837)	118.3 (816)		
CM	2.5 (801)	36.9 (812)	98.9 (839)		
BS	4.5(1850)	40.1(1938)	67.1(1336)		
JD	7.1(1495)	44.3(1734)	107.8(2103)		
Mean ±1 SD	4.4 ± 2.6	45.2 ± 4.9	96.2 ± 15.5	147.8 ± 9.1	208.9 ± 14.0

Numbers in parenthesis refer to the total number of CB cells scored.

duces micronuclei, the numbers of micronuclei in unirradiated cultures from 4 individuals were measured both in conventional cultures and in Cyt-B cultures. In order to compare the two sets of observations it was necessary to correct for the fact that each CB cell arrested by Cyt-B would otherwise have given rise to two mononuclear interphase cells. Therefore, for the Cyt-B cultures the expected number of micronuclei/1000 notional cells was given by:

$$(C \times M_c) + (I \times M_i) \times \frac{1000}{1000 + C} \qquad \text{(a)}$$

where C = number of CB cells per 1000 cells, M_c = micronuclei per CB cell, I = number of interphases in 1000 cells, and M_i = micronuclei per interphase.

The measurements were made at 72 h and the results are shown in Table 2. There was no difference between the number of micronuclei/1000 cells as estimated by the standard method and the number of micronuclei/1000 notional cells as estimated by the CB method, thus indicating that Cyt-B does not induce micronuclei.

Finally, a comparison was made between the data obtained by the autoradiographic method and the CB method for the number of micronuclei induced by 100 and 200 rad of X-irradiation. Because CB cells would have otherwise divided into two cells which would have shared the micronuclei, a comparison between the two methods could be made by plotting the results as micronuclei per 500 CB cells and micronuclei per 1000 labelled cells. In each case the base-line levels of micronuclei in the unirradiated cultures were subtracted; in the case of the autoradiography results the data from the plateau region between 72 and 84 h were averaged. The comparison is shown in Fig, 7 which indicates that the dose–response results from the two methods were in close agreement.

Discussion

It is important to develop simple and reliable techniques for biological dosimetry of exposure to radiation or other forms of genotoxic agents because such exposure is potentially carcinogenic and genotoxic (Klein, 1981; Yunis, 1983). The micronucleus method as applied to the lymphocyte culture system has the potential for becoming such a technique since measurement of the baseline number of micronuclei is simple and rapid and can be used for population screening. The method also has the potential to enable quantitation of micronuclei following exposure of lymphocytes to genotoxic agents in vitro, provided however that the baseline number of micronuclei can be subtracted from the number expressed after genotoxic exposure.

Unfortunately the establishment of base-line levels and induced levels of micronuclei cannot be performed until the kinetic problem of micro-

TABLE 2

A COMPARISON OF BASE-LINE LEVELS OF MICRONUCLEI IN THE STANDARD METHOD AND CYTOKINESIS-BLOCK (CB) METHOD

Subject	Standard method	CB method				
	Micronuclei/ 1000 cells	CB cells/ 1000 cells	Micronuclei/ CB cell	Interphases/ 1000 cells	Micronuclei/ interphase [a]	Micronuclei/ 1000 notional cells [b]
S	7.0	130	0.009	870	0.000	1.0
JD	6.0	525	0.014	415	0.002	5.4
MF	4.0	210	0.016	790	0.001	3.4
BK	3.0	225	0.009	775	0.004	5.1
Mean ± 1 SD	5.0 ± 1.6					3.7 ± 1.7

[a] 1000 interphase cells were scored.
[b] Estimates were calculated by using Eqn. (a).

nucleus expression is recognised. Micronuclei can only be expressed if cells divide. Lymphocytes from different individuals respond quite differently to PHA so that the proportion of dividing cells in culture differs from individual to individual. Even in cultures from the one individual, the proportion of lymphocytes which have undergone only one division will be very dependent on culture conditions and the time at which cultures are terminated. Pincu et al. (1984) also recognised the kinetic problem and developed a method which was based on the same principle as the autoradiographic method. In their technique lymphocytes were enabled to continuously incorporate a large amount of bromodeoxyuridine so that incorporating cells could be subsequently recognised by differential Giemsa staining. However, their method has the disadvantage that (a) cells in late S or G_2 of the first cycle are scored in addition to cells in the second cycle (b) the large dose of BrdU may itself produce chromosomal alterations as evidenced by production of sister-chromatid exchange and possibly chromosomal breaks (Mazrimas and Stetka, 1978), and micronuclei (our unpublished observations and Sudharsan and Heddle, 1980).

The autoradiographic method described in this paper enables quantitation of micronuclei in a synchronised sub-population of lymphocytes. This method, however, is laborious and it cannot be used for estimation of base-line levels of micronuclei because the incorporated tritiated thymidine itself induces micronuclei presumably due to the emission of β particles. However the method can be used for measuring induced levels of micronuclei if the measurements are made on the plateau region as shown in Fig. 4. Thus for measuring micronuclei induced by X-irradiation, the autoradiographic method gave identical results to the cytokinesis-block method.

In the cytokinesis-block method, enumeration of micronuclei is restricted to cells that are blocked from undergoing cytokinesis and which are consequently easily recognisable as large binucleate cells. This method is feasible because it is possible to accumulate CB cells by pulsing cultured lymphocytes with Cyt-B, an inhibitor of cytokinesis, an approach which is analogous to the use of colchicine to accumulate cells in metaphase for chromosomal analysis. The biological effects of Cyt-B were first described for L cells by Carter (1967, 1972) who showed that Cyt-B at low concentrations (1 μg/ml) inhibited cytoplasmic cleavage, apparently without blocking mitosis and the same effect on lymphocytes has been observed by a number of other workers (Ridler and Smith, 1968; Wright and Hayflick, 1972; O'Neill, 1972; Kelly and Sambrock, 1973). Normal lymphocytes cultured in the presence of Cyt-B may show a few tetraploid and octoploid metaphases but chromosomal abnormalities have not been observed (Ridler and Smith, 1968; O'Neill, 1972). Neoplastic cells may show more marked effects including persistant nuclear division and chromosomal pulverization. Based on these data we investigated the use of Cyt-B as a method for accumulation of binucleate cells. The final technique involved Cyt-B at a concentration of 3.0 μg/ml being continuously present between 48 and 72 h of culture and under these conditions there was no evidence that Cyt-B itself caused an increase in micronculei. Although the great majority of micronuclei were observed in CB cells, a few were observed in mononuclear interphase cells, perhaps due to a few cells dividing before addition of Cyt-B or a few cells escaping the Cyt-B block. However, the fact that not all cells were accumulated does not bias the results unless those that were blocked preferentially over- or under-expressed micronuclei, which seems unlikely.

Of the two methods the cytokinesis-block method is clearly the superior. CB cells are at an ideal stage for scoring micronuclei because they are certain to have divided and micronuclei can still be easily recognised. The levels of CB cells accumulated will depend on the proportion of cells in culture that respond to mitogen but it is generally possible to accumulate several hundred CB cells from a 1-ml culture. Since Cyt-B does not induce micronuclei or chromosomal damage the CB method can be used both to measure the base-line level of micronuclei, which reflects in vivo chromosomal damage, and to allow comparisons of mutagen-induced micronuclei in individuals whose lymphocytes might respond differently to **PHA.** Furthermore, by scoring micronuclei in CB cells, a given amount of work, which is required to score a given number of cells, will result

in enumeration of twice the number of micronuclei and will thus lead to increased precision of the final estimate. For these various reasons the CB method appears to be a simple and reliable method for quantitation of micronuclei in lymphocytes.

References

Carter, S.B. **(1967)** Effects of cytochalasins on mammalian cells, Nature (London). **213. 261–264.**

Carter. S.B. **(1972)** The cytochalasins as research tools in cytology, Endeavour, **31, 77–82.**

Countryman, P.I., and J.A. Heddle **(1976)** The production of micronuclei from chromosome aberration in irradiated cultures of human lymphocytes, Mutation Res., **41, 321–332.**

Heddle, J.A., B.L. Constance, E.F. Saunders and D. Benz **(1978)** Sensitivity to five mutagens in Fanconi's anaemia as measured by the micronucleus method, Cancer Res., **38,** 2983–2988.

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

Kelly. F., and J. Sambrock **(1973)** Differential effect of cytochalasin B on normal and transformed mouse cells, Nature (London) New Biol., **242, 217–219.**

Klein. G. **(1981)** The role of gene dosage and genetic transpositions in carcinogenesis, Nature (London), **294, 313–318.**

Mazrimas, J.A., and D.G. Stetka **(1978)** Direct evidence for the role of incorporated BUdR in the induction of sister chromatid exchanges, Exp. Cell Res., **117, 23–30.**

O'Neill. **F.J. (1972)** Chromosome pulverisation in cultured normal and neoplastic cells treated with cytochalasin **B,** J. Natl. Cancer Inst., **49,** 1733–1737.

Pincu, M., D. Bass and A. Norman (1984) A improved micronuclear assay in lymphocytes, Mutation Res., **139, 61–65.**

Ridler. M.A.C., and G.F. Smith **(1968)** The response of human cultured lymphocytes to cytochalasin B, J. Cell. Sci., **3, 595–602.**

Sudharsan Raj, A., and **J.A.** Heddle **(1980)** Simultaneous detection of chromosomal aberrations and sister chromatid exchanges, Experience with DNA-intercalating agents, Mutation Res., **78, 253–260.**

Wright, **W.E.,** and L. Hayflick **(1972)** Formation of a nucleate and multinucleate cells in normal and SV40 transformed **WI-38** by cytochalasin, B, Exp. Cell. Res., **74, 187–194.**

Yunis. J.J. **(1983)** The chromosomal basis of human neoplasia, Science, **221, 227–235.**