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Benzene-Induced Myelotoxicity: Application of Flow Cytofluorometry for the Evaluation of Early Proliferative Change in Bone Marrow

by Richard D. Irons*

A detailed description of flow cytofluorometric DNA cell cycle analysis is presented. A number of studies by the author and other investigators are reviewed in which a method is developed for the analysis of cell cycle phase in bone marrow of esperimental animals. Bone marrow cell cycle analysis is a sensitive indicator of changes in bone marrow proliferative activity occurring early in chemically-induced myelotoxicity. Cell cycle analysis. used together with other hematologic methods, has revealed benzene-induced toxicity in proliferating bone marrow cells to be cycle specific, appearing to affect a population in late S phase which then accumulate in G_2/M.

Introduction

Historically, the toxicologic evaluation of myelotoxicity in experimental animals has been largely limited to classical hematologic evaluation of peripheral blood elements and erythrocytic indices. However, due to the number of peripheral factors which govern and serve to buffer the size of the circulating blood cell pool, hemopoiesis is more accurately evaluated in the context of bone marrow proliferative activity. Alterations in blood precursor cell growth and maturation represent some of the earliest and most subtle alterations occurring in myelotoxic response, often preceding measurable changes in cell numbers or morphology by hours or even days. The application **of flow** cytofluorometry for **DNA** cell cycle phase analysis grew out of a need to develop a more sensitive and direct means to examine the cell cycle dynamics of proliferating bone marrow cells.

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Flow cytofluorometry is a relatively new instrumental technique which permits the quantitative measurement of a variety of properties on individual cells in a flow stream at rates of several thousand cells per minute. The first cytometer was introduced by Kamentsky in 1965 (*1*), and only in the last few years have multiparameter cell sorters and cytofluorometers become available for use by the biologist. Flow cytometer, flow microfluorometer, **flow** cytofluorometer, fluorescence activated cell sorter and laser-based multiparameter cell sorter are all terms which have been used interchangeably to describe similar types of instrumentation. Examples **of** measurements which can presently be made on individual cells include: single and dual wavelength fluorescence, low-angle light scatter (related to cell size), cell number, cell volume, and a variety **of** other experimental parameters (*2-4*).

Flow cytofluorometry has been used in a variety of applications in biology which have been reviewed by Horan and Wheeless (*3*). The application of flow cytofluorometry for cell cycle analysis is based upon the capability to measure **DNA** content in individual cells. Prior to analysis cell suspensions are

stained with fluorescent compounds which stochiometrically bind to DNA and for which staining intensity is proportional to DSA content. The intensity of fluorescence can be used to measure individual cell DNA content and in turn to estimate the proportion of the total cell population in each phase of the cell cycle (5). Examples of staining reagents which have been used for this purpose include the interchalating dyes ethidium bromide and propidium iodide (6-8) and the so-called "groove" binding antibiotics, mithramycin(9) and chromomycin (10). The principal advantages offered by cytofluorometry for the study of proliferating populations include the ability to look at individual cells, speed, sensitivity, and the information the technique provides with respect to alterations in cell cycle distribution among dividing precursor cells. It cannot presently discriminate among different cell lines, nor does it provide direct information on changes in the rate of **DNA** synthesis. Thus, in its present state of development, flow cytofluorometric DNA cell cycle analysis complements but does not replace other methods for evaluating bone marrow cell populations such as ^{3}H-TdR/DNA turnover, stem cells (CFU-S) or precursor cell (CFU-C, BFU-E) assays.

Principles of Instrumentation and DNA Cell Cycle Analysis

The basic design of the cytofluorometer is similar to that of a fluorometer, the three primary components being a source of excitation, a means of sample introduction or containment, and a detector (Fig. 1). **A** suspension of single cells is introduced by means of a laminar flow chamber into a stream adjusted so that the cells pass through in single file. High efficiency excitation is required to provide the sensitivity necessary to measure fluorescence in individual cells. This is achieved in most instruments by the use of a laser light source although mercury-arc lamps are also used. Fluorescence detection is accomplished by using photoelectric detectors placed perpendicular to the exciting light beam. Specificity is obtained by using monochromatic laser light of a particular exciting wavelength and appropriate barrier filters. An additional feature of cell sorters is the ability to electrostatically separate cells on the basis of their light scatter or fluorescence characteristics.

The landmark work of Howard and Pelc in the early 1950's heralded the now widespread use of radiotracer techniques to study DNA synthesis and forms the basis of our current concept of the DNA cell cycle (11). Relative to DNA synthesis the cell

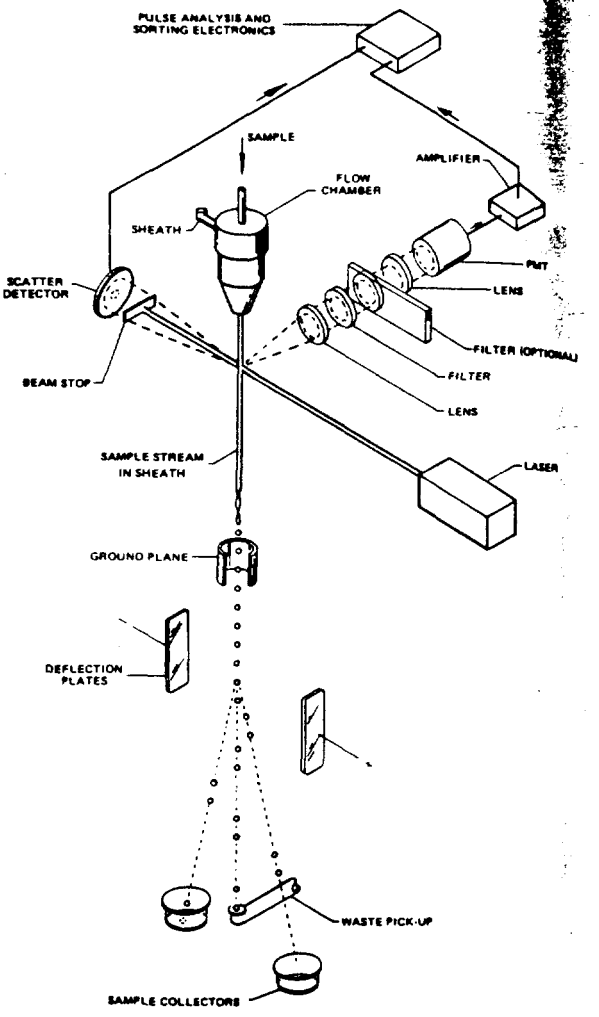


FIGURE 1. **Schematic diagram of the** TPS-I **laser based cell sorter. Courtesy Coulter Electronics. Inc.. Hialeah. Fla.**

life cycle can be divided into four discrete phases: M, mitosis, or physical cell division; G_1, the first gap or period of **no** measurable DNA synthesis immediately following division; S, the phase of measurable DNA synthesis; followed by G_2, the second **gap** after DNA synthesis is complete, during which the cell contains twice the diploid content of DNA found in G_1 (Fig. 2). An additional phase, G_0, is often used to denote cells, usually indistinguishable from G_1 cells on the basis of DNA content, which are not actively cycling.

Normal bone marrow contains a heterogeneous population **of** precursor cells which can be characterized as undergoing asynchronous exponential growth, i.e., proliferating cells move through the cell cycle independent of one another and the transit time for passage through any given phase of

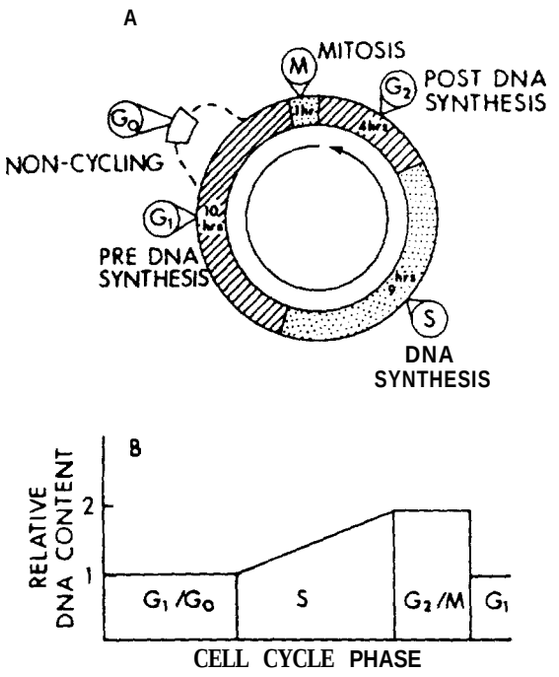


FIGURE 2. Schematic representation of (*A*) DNA cell cycle phase based on a hypothetical 24-hr cycle; (*B*) relative DNA content of the cell as a function of cell cycle phase.

the cycle is independent of the transit time through any other phase. For any given point in time, the probability that a cycling cell will be in a particular phase of the cell cycle is proportional to the amount of the total cell cycle transit time that is spent in that phase. In a theoretical DNA distribution histogram for a cell population in asynchronous exponential growth, G_1/G_0 and G_2/M phases are represented by single values for DNA content, the latter being exactly twice the value of the first (Fig. 3*A*). S phase is represented by a linear continuum of values. An actual DNA distribution histogram obtained from normal rabbit bone marrow illustrates the contribution of instrumental instability, lack of proportionality between DNA content and fluorescence intensity and biologic variability to experimental error. A gaussian distribution of points about a modal value define G_1/G_0 and G_2/M phases (Fig. 3*B*).

It is now generally accepted that the phases of the cell cycle are not homogeneous and can further be divided into any number of compartments of subphases on the basis of additional criteria such as RNA synthesis, protein synthesis, enzyme activities, or even differences in the rate of DNA synthesis between early and late S phase (*12*, *13*). These reservations not withstanding, it is possible

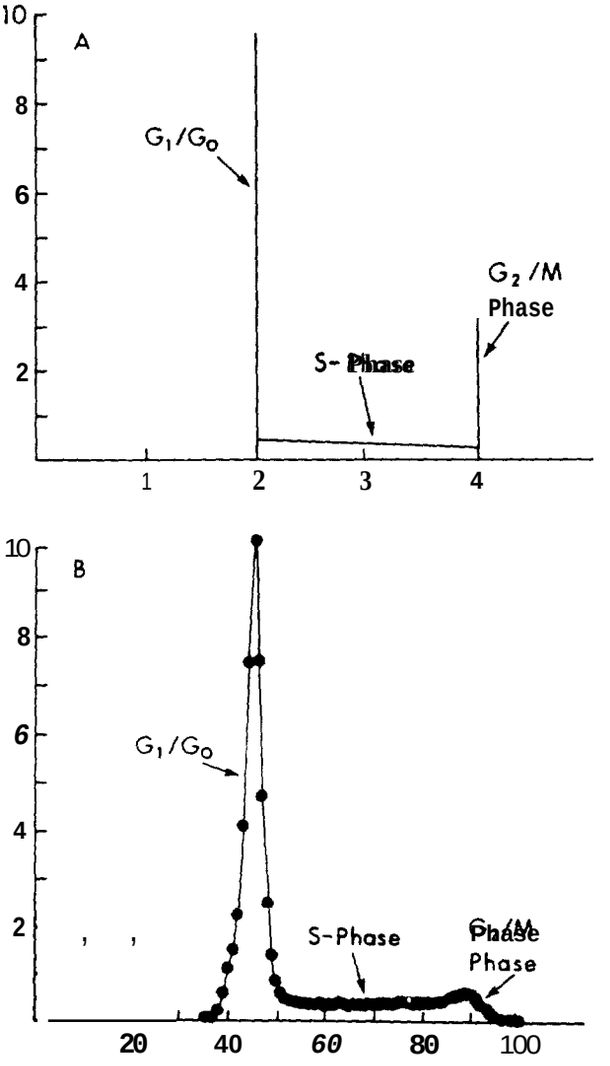


FIGURE 3. DNA distribution histograms: (*A*) theoretical DNA distribution histogram for a population of cells in asynchronous growth; (*B*) actual DNA distribution histogram obtained from cytofluorometric analysis of normal rabbit bone marrow.

to monitor the effects of cytotoxic agents on cell growth and maturation by measuring the DNA cycle distribution of proliferating cell populations.

Sample Preparation

Flow cytofluorometric analysis requires monodisperse cells in suspension. For blood and bone marrow, tissue dispersion does not represent a problem and very satisfactory results can be achieved by mechanical aspiration. In order to improve sensitivity and to insure uniform samples of cells it

is desirable to separate potentially cycling and cycling precursor cells from mature granulocytes and erythrocytes. A relatively small proportion (20-50%) of the normal bone marrow cell population is made up of potentially cycling cells. The remainder are mature or maturing precursor cells in the late stages of differentiation which have lost the capacity to divide. These mature cells represent a significant and highly variable proportion of the bone marrow cell population. Mature cells, excluding erythrocytes, normally contain a diploid complement of DNA and therefore contribute to the number of cells in the G_1/G_0 region of the histogram. Furthermore, the normal cycling and potentially cycling bone marrow precursor cell population contains less than half of the cells in S, G_2, and M at any one time (18 to 35% in species examined). The inclusion of mature nondividing cells in the sample would result in approximately 95% of the cells appearing in G_1/G_0, greatly reducing the sensitivity of cell cycle phase analysis. For this reason, and to remove dead cells, nuclear debris. and other stainable material not associated with cycling cells, it is advantageous to provide a reproducibly enriched population of precursor cells for DNA analysis.

Most physical separation procedures for cells are based on differences in cell sue or density. Elutriation and Sedimentation velocity methods separate cells on the basis of size. These methods are particularly useful for the preparative isolation of large numbers of cells. Although these have been used successfully for the separation **of** blood and bone marrow cells (*14*, *15*), they are somewhat cumbersome and inefficient for the routine separation of small bone marrow samples. These difficulties arise partly from the relatively narrow size distribution among bone marrow cells (*16*). Buoyant-density methods rely on differences in the density of cells. Density separation or isopycnic sedimentation usually employ media of different densities to collect cells at interfaces equivalent to their buoyant

In preliminary studies of the application **of** flow cytofluorometry for the analysis of cell cycle phase in experimental animals, we isolated bone marrow precursor cells employing the widely used isopycnic

to

of ^{3}H-TdR labeled bone marrow precursor cells in S phase. The optimum density for the separation of rabbit and rat bone marrow precursor cells was found to be **1.077** g/cm^3, enabling the use of a single **step** gradient to obtain a reproducibly enriched population of bone marrow precursor cells (*18*). Morphologic examination of isolated bone marrow cell fractions indicate a fourfold enrichment of potentially cycling cells (Table 1).

DNA-Histogram Analysis

Several methods have been used to obtain quantitative estimates of the relative size of the various cell cycle phase compartments from **DNA histo**grams. These methods range in complexity from manual calculation of the proliferative index (PI)to computer based iterative least squares fitting techniques.

The determination of PI is the simplest method to obtain an indication of synchrony in a population **of** cycling cells. It provides a single parameter for comparison of DNA histograms that is proportional to the percentage of cycling cells in the population Rigorously defined, PI should be the percentageof cycling cells in the total cell population (i.e., G_1, S, G_2, and M). However, since it is not possible to distinguish between G_1 and G_0 cells simply on the basis of DNA content, G_1 cells are not included in

Table 1. Purification of bone marrow precursor cells by isopycnic sedimentation.

Stage of maturation	Bone marrow suspension[a]	Cell fraction Ficoll-DMEM interface[a]
(Proerythroblasts, myeloblasts. undifferentiated blasts. lymphocytes)	6.5 ± 1.4	60.3 ■10.3
Dividing precursors (basophilic erythroblasts. promyelocytes. myelocytes)	14.5 ± 2.3	13.5 ± 5.5
Late precursors and mature cells (polychromatophilic erythroblasts. metamyelocytes. bands. segmented pseudoeosinophils)	81.5 ■2.8	18.8 ± 8.3
Total potentially cycling cells (a and b)	21.0 ■3.2	81.3 ± 8.31

'Values espressed as means of percent ± SEM of total cells for four individual preparations. Rabbit marrow cells were suspended in Delbecco's modified Eagle's medium (DMEM), centrifuged over fetal calf serum to remove debris. anti layered over Ficol hypaque (δ = 1.077 g/cm^3). Followng centrifugation for 30 min at 500g cells at the Ficoll-DMEM interface were collected and washed. Wright-stained cytocentrifuge slide preparations were made of hone marrow suspensions and Ficoll-separated cell fractions which were then submitted to differential morphologic evaluation. Modified from Horan et al. (*18*).

the numerator and PI becomes a conservative estimate of the cycling cell population:

$$PI = \frac{\text{Number of cells in S, } G_2\text{, and M}}{\text{Total number of cells counted}}$$

The advantages of PI as a measurement are that it is simple to perform and provides a single term for comparison of different cycling populations (Fig. 4). The principal disadvantage of PI is that it imparts no information on the distribution of cycling cells about the cell cycle phase and can often be misleading. A case in point is a comparison of the effects of benzene and bromobenzene on synchrony in bone marrow. The PI of bone marrow of normal rabbits varies from animal to animal but averages approximately 35%. An increase in PI is observed following short-term administration of benzene (PI = 65%) or bromobenzene (PI = 48%); however, even a visual examination of DNA histograms obtained from these animals reveals a marked difference in the DNA distribution of bone marrow cells following exposure to these two agents (Fig. 5). Benzene administration is accompanied by a relative accumulation of cells in the G_2/M region of the DNA distribution histogram, whereas bromobenzene exposure is associated with a broad accumulation of cells at the G_1-S interface.

Two computor based algorithms are currently available for parametric analysis of DNA histograms (*19-21*). Both methods fit the G_0/G_1 and G_2/M peaks to single Gaussian curves but differ in the function used to estimate the size of the S-phase population. The algorithm of Fried approximates the S phase portion of the histogram to a series of evenly spaced Gaussian curves. Alternatively, the method of DeanJett utilizes an expanded second order polynomial for the same purpose (Fig. 6). As with all curve fitting or stripping techniques which are used in compartmental analysis. criteria for selection of an appropriate method for analysis are largely empirical, the choice usually being based on the precision with which the method approximates the original data.

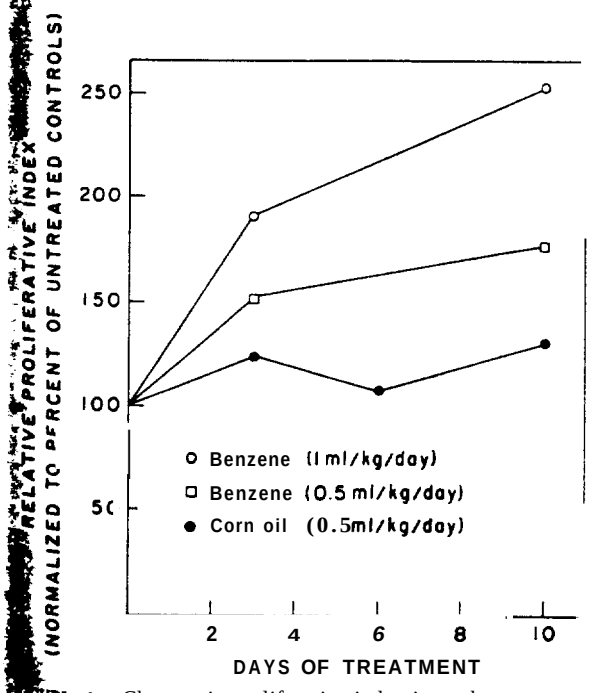


FIGURE 4. Changes in proliferative index in rat bone marrow following pretreatment with benzene.

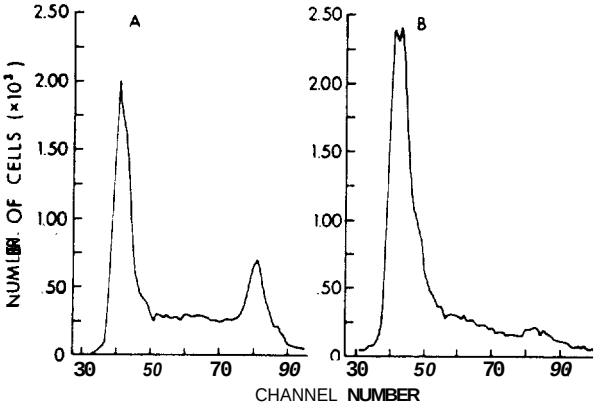


FIGURE 5. DNA histograms of (*A*) rabbit bone marrow obtained on day 6 after repeated administration of benzene (0.5 ml/kg on days 0. 2. and 4); (*B*) rabbit bone marrow following administration of bromobenzene following the same protocol. Propidium iodide stain.

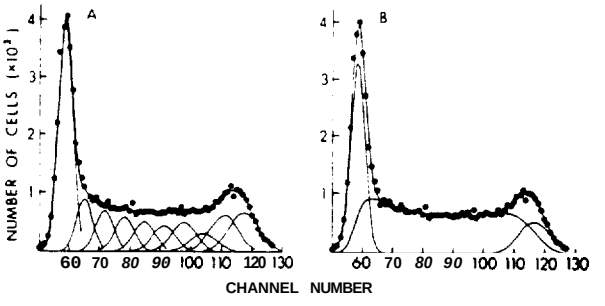


FIGURE 6. Computer-generated fits of DBA distribution histograms obtained from mithramycin stained L5178Y cells: (*A*) Fried Algorithm using a series of multiple Gaussian curves to estimate S phase: (*B*) DeanJett algorithm using an expanded second order polynomial to estimate S-phase. (*) actual raw data; (——) computer-generated fit. Data taken from Scheck et al. (*27*).

Cytofluorometric Analysis of the Effects of Myelotoxic and Chemotherapeutic Agents on Bone Marrow Cell Cycle Phase

A substantial body of literature has established that cytotoxic agents may block or delay passage of proliferating cells through the cell cycle (*22*). Cells may demonstrate variable sensitivity to many cytotoxic agents according to their position in the cell cycle; the phase of maximum sensitivity may or may not correspond to the phase of arrest or cell death. Although somewhat of an oversimplification, cytotoxic agents may be classified as cycle-specific, affecting cells only in active cycle (sparing G_0 cells); phase-specific, affecting cells in a given phase of the cell cycle; or cycle-nonspecific, affecting cells independent of their position in the cell cycle. Exposure of an asynchronous population of dividing cells to an agent which is cytotoxic or blocks progression at a particular phase in the cell cycle can result in a surviving cell population which is partially synchronizecl or synchronized. This can be brought about either by an accumulation of cells at the region of the block, the surviving population of cells having been spared based on their position in the cell cycle at the time of exposure, or both.

Cytofluorometric analysis is extremely sensitive. revealing perturbations in asynchronous growth patterns that may represent only a small portion of the proliferating cell population. However, because it is now always possible to distinguish among alternative explanations of cycle distribution changes, final interpretation of cell cycle phase analysis *in* vivo may require additional information concerning changes in bone marrow cellularity. rate of cell turnover, or specific precursor cell function. *In vitro* studies on the behavior of cells in culture are often helpful as well. Both the complesity and the sensitivity of cytofluorometric analysis are best illustrated by example. Changes in cell cycle distribution in rat bone marrow following demecolcine administration are demonstrable as early as one hour after administration, at which time an accumulation of cells in the G_2/M region is observed (Fig. 7). Although the block is more prominent 4 hr after administration, the leukocyte count is still essentially normal. Colchicine and clemecolcine are examples of mitotic spindle poisons which are phase specific, blocking cells at mitosis. Sensitivity to these agents is primarily restricted to S phase, during which period they bind to microtubular protein prior to assembly (*23*). Not all cycling cells are affected, accounting for the partial but not total synchronization observed *in vivo*. In contrast to colchicine vinblastine presents a less straightforward pattern. Cell cycle analysis of rabbit bone marrow 2 hr following vinblastine administration reveals an early reduction in the relative numbers of cells in S and M; however, at 6 hr an accumulation in G_2/M is observed (Fig. 8). The DNA distribution histogram obtained from rabbit bone marrow following vinblastine administration in *vivo* could be the subject of a variety of interpretations in the absence of data on bone marrow cellularity or knowledge of the effects of vinblastine on synchronized cells in culture. It is known, however, that, in addition to binding to microtubulin, vinblastine possesses antimetabolic activity as well. Cells in culture exposed to vinblastine in S-phase proceed to mitosis and accumulate, whereas cells exposed in late G_1 phase undergo lysis prior to mitosis (*22, 23*). The *in vivo* findings mirror those obtained *in vitro* and reflect two simultaneous cycle specific phenomena.

The first application of flow cytofluorometric cell cycle analysis for the toxicologic evaluation of an industrial compound has been the study of experimental benzene toxicity. The relationship between chronic benzene exposure and myelotoxicity has been recognized for over 70 years. A variety of

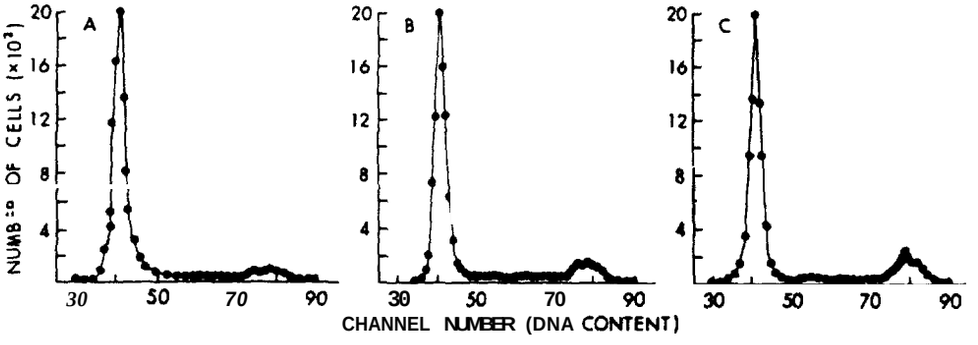


FIGURE 7. **Bone marrow DNA distribution histogram obtained from Fischer-344 rats following single** IP **administration** of **demecolcine.** 1 mg·kg; (*A*) 0 **time:** (*B*) 2 hr: (*C*) 4 **hr. Mithramycin stain.**

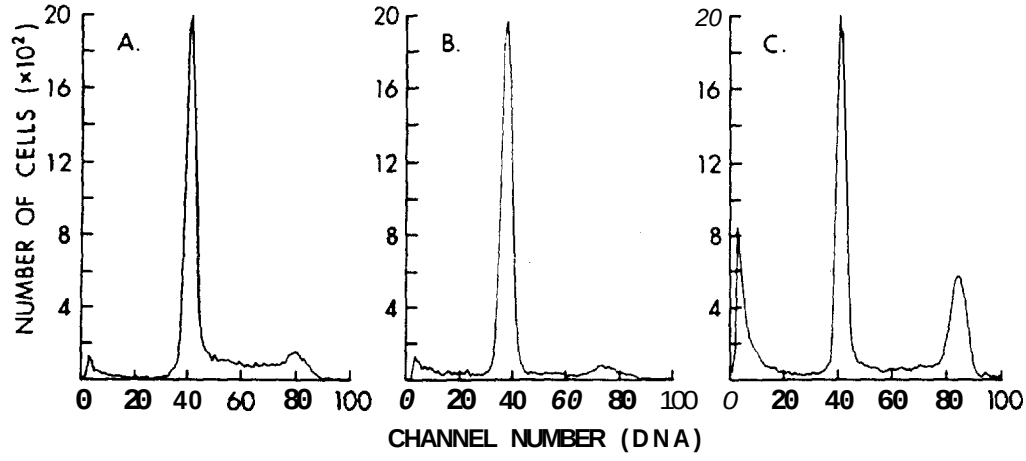


FIGURE 8. **Bone marrow DNA distribution histograms obtained** from rabbit following **administration** of **a single dose of vinblastine (2 mg/m²): (A) 0 time:** (*B*) 2 hr: (*C*) 6 hr. Propidium **iodide stain.**

blood dyscrasias have been observed in man and experimental animals; however, studies vary widely **as** to the specific effects of benzene and the conclusions drawn **as** to the most sensitive parameter or cell line. **Various** investigators have reported anemia, leukopenia, thrombocytopenia, lymphocytopenia, leukemia, bone marrow hyperplasia or hypoplasia, **asplastic** anemia and, most recently, hemolymphoreticular neoplasia **as** occurring singly or in combination **as a** consequence of chronic benzene exposure.

Additional studies have reported chromosomal abnormalities occurring in bone marrow, including mitotic arrest, inhibition of thymidine incorporation into promyelocytes and basophilic and polychromatophilic erythroblasts, and megaloblastic changes in the erythrocytic series. These findings suggest that benzene produces a defect in maturation of proliferating bone marrow cells that does not appear to be restricted to any single cell line. An especially comprehensive review on the subject is that by Laskin and Goldstein (*24*).

Hematologic examination of the peripheral blood in the rat following repeated short-term administration of benzene reveals a dose-dependent depression in the number of circulating leukocytes, lymphocytes demonstrating a particular sensitivity *(25).* Erythrocytes and erythrocytic indices remain within normal limits as would be expected based on the half-life of the circulating erythrocyte and the time frame of these experiments (Fig. 9). The rabbit exhibits a similar lymphocyte depression in response to benzene esposure: however. it appears to be about twice **as** sensitive (Table 2) (*26*).

Cell cycle phase analysis of bone marrow in the rat reveals alterations in cell cycle distribution similar in type and magnitude to those observed with demecolcine; although the dose and duration of exposure required to produce a comparable effect are much greater for benzene than demecolcine (Fig. 10). Compartmental analysis of DNA histograms using the Fried algorthm reveals a dose-dependent accumulation of cells in the G_2/M region which is eventually reflected by a decrease in G_1/G_0

Table 2. Effects of repeated subcutaneous benzene administration on circulating

	Lymphocytes mm⁻³ blood[b]		
Day	Corn **oil**	**Benzene** (0.25 ml kg day)	**Benzene** (0.5 ml kg day)
0	3051 ± 128 (23)	3320 ± 239 (12)	3051 ± 128 (23)
1	2975 ± 199 (6)	2929 ± 326 (8)	3127 ■441 (9)
3	3190 ± 227 (6)	—	2043 ± 326 (7)
5	3324 ± 342 (6)	2174 ± 353 (8)	1153 ± 193 (7)
10	3590 ± 573 (6)	2113 ■404 (8)	701 ± 210 (6)
28–33	3334 ± 153 (4)	2568 ± 410 (4)	1410/2755 (2)
40	—	2730 ± 1056 (4)	—

[a]Rabbits were treated daily for 10 days and allowed to recover. Data taken from Irons and Moore (*26*).
[b]Means ± 1 SE for (*n*) animals.

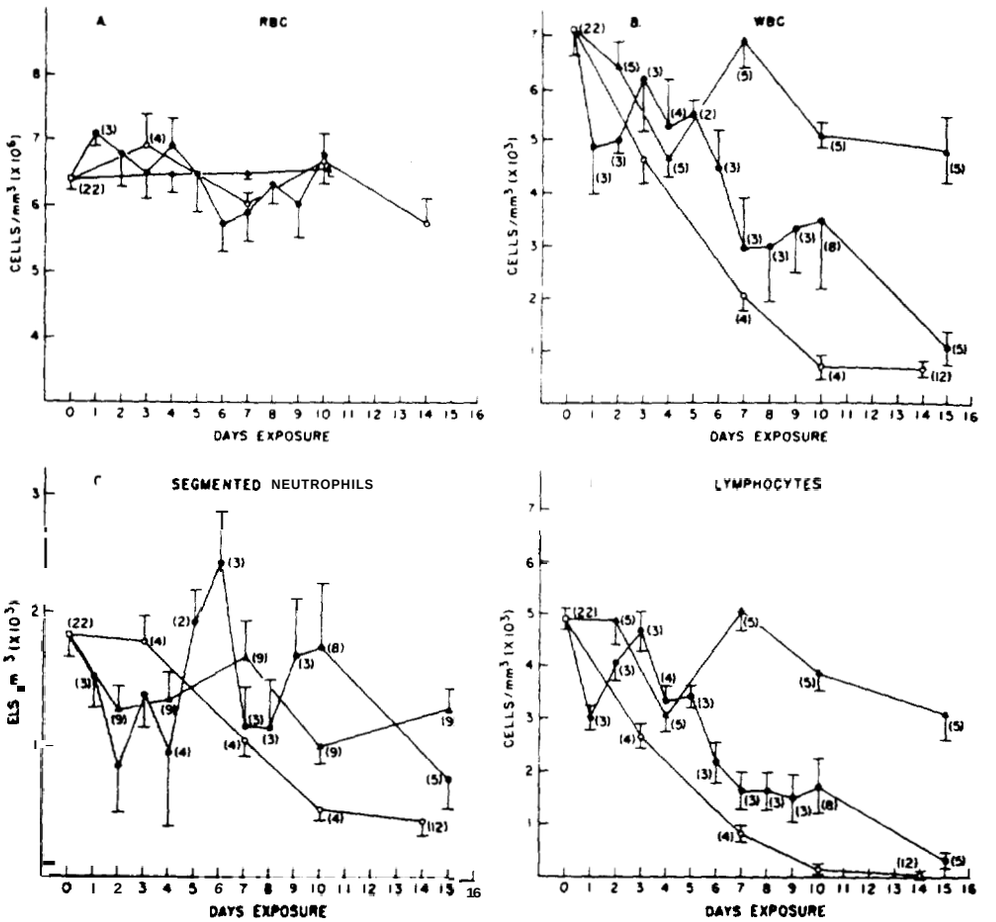


FIGURE 9. Absolute numbers of circulating cells in the peripheral blood following repeated exposure to benzene. From Irons et al. (25).

Table 3. Compartmental analysis of DNA histograms obtained from rat bone marrow.

Treatment group	% of total cells in compartment[a]		
	G_0/G_1	S	G_2/M
Untreated	82.9 ± 0.81	12.1 ± 0.38	5.11 ± 0.55
Corn oil 0.25 mg/kg/day (for 10 days)	82.0 ± 1.59 NS[b]	13.0 ± 0.86 NS[b]	5.01 ± 0.76 NS[b]
Benzene 0.5 mg/kg/day (for 10 days)	79.2 ± 2.08 NS[c]	14.0 ± 1.78 NS[c]	6.79 ± 0.35 $0.025 < p < 0.06$
Benzene 1.0 mg/kg/day (for 10 days)	73.1 ± 1.06 $0.005 < p < 0.01$	15.0 ± 0.69 NS[c]	11.6 ± 0.76 $0.005 < p < 0.01$
Bromobenzene 0.5 mg/kg/day (for 10 days)	87.4 ± 1.53 $0.025 < p < 0.05$	9.0 ± 1.61 NS[c]	3.63 ± 0.24 NS[c]

[a]Values represent mean ± SD for three animals, Compartmental analysis was obtained from computer fit of DNA histograms. Values for S represent the sum of five compartment derived by the Fried algorithm as described in Methods. Data taken partially Irons et al. (25).

[b] = No significant difference from untreated ($p > 0.05$).

[c]NS - No significant difference from corn oil treated ($p > 0.05$). Benzene treated groups are compared to corn oil controls.

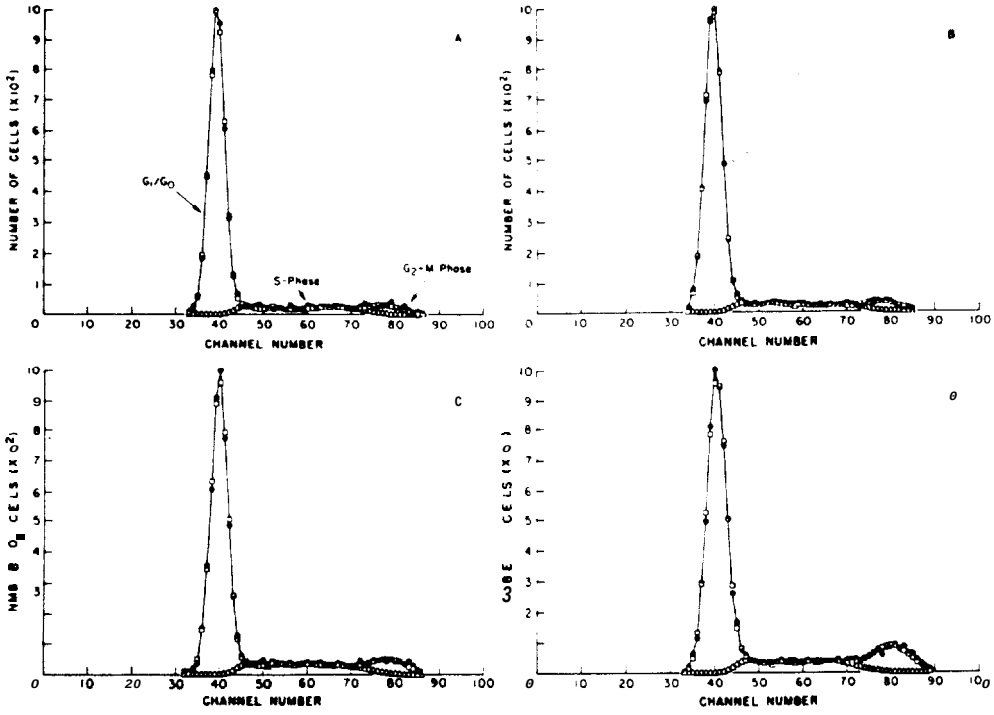


FIGURE 10. **Bone marrow DNA distribution histograms obtained from** Fischer-344 **rats** (*A*) **untreated:** (*B*) **following subacute administration** of **corn oil,** 0.5 ml kg; (*C*) **following administration of benzene.** 0.5 **ml/kg/day;** (*D*) **following administration of benzene. 1.0**ml/kg/day for 10 days: (●) raw histogram data; (—) **computer fit;** (Δ) **S-phase compartment approximated by Fried** Algorithm. **Reproduced from Irons et** al. **(25).**

cells (Table **3).** Changes in bone **marrow** cellularity **are** also observed but are relatively constant over **the** same period of time, ranging from 80% to 65% **of** normal, depending on the dose (Fig. 11).

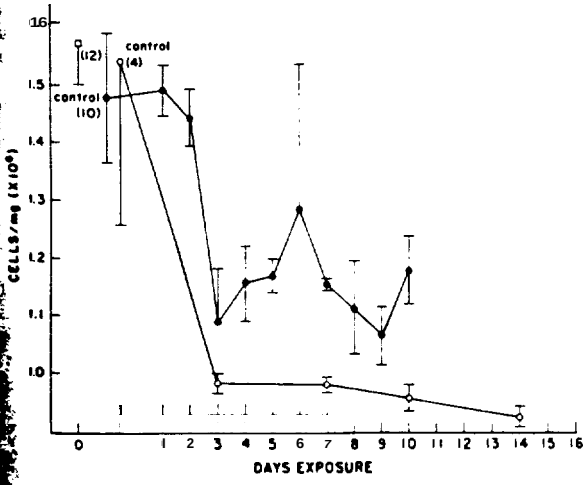


FIGURE 11. Bone marrow cellularity during repeated benzene administration. Reproduced from Imns et al. (*25*).

Following DNA turnover in rat bone marrow using ^{3}H-TdR we found the specific activity of **DNA** to be increased **2** and **4** hr following pulse labeling but normal there afterwards, the temporal sequence suggesting the possibility of the loss of cells labeled in late S phase (Fig. **12).**

These findings suggest that benzene induces changes in bone marrow cell cycle distribution and maturation which are similar to those observed with colchicine, affecting a sensitive population of cells in late S phase which then accumulate in G_2 or M. Differential examination of bone marrow smears reveals some depression in rapidly dividing precursor cells including: promyelocytes, myelocytes, basophilic and polychromatophilic erythroblasts as well **as** lymphocytes with little change in mature nondividing cell types such **as** metamyelocytes, bands, and neutrophils(*25*).

Structure-activity relationships are an important consideration in the expression of bone marrow toxicity to benzene. Bromobenzene does not elicit the same alterations in bone marrow cell cycle activity in either the rabbit (Fig. *5)* or the rat (Table **3).** Neither does administration of bromo-

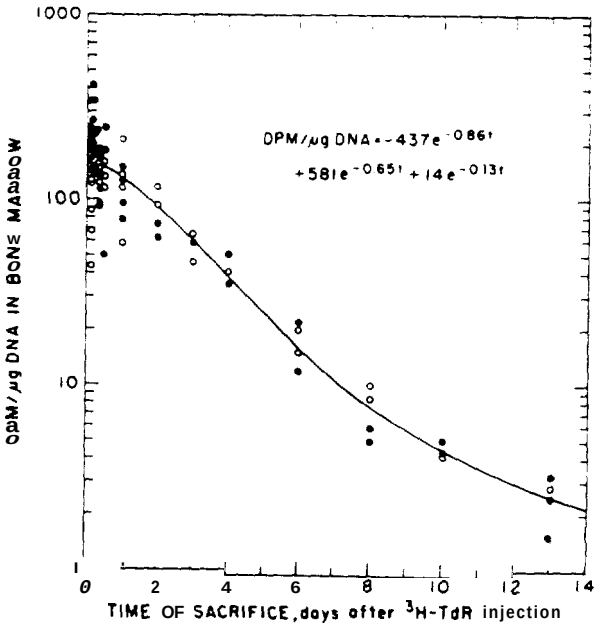


FIGURE 12. Decrease in specific activity of bone marrow DNA following pulse labeling with ^{3}H-TdR. Reproduced from Irons et al. (25).

benzene produce a reduction in peripheral blood leukocytes in these species. The rabbit demonstrates a much more pronounced accumulation in G_2/M following a much smaller dose and duration of exposure to benzene than does the rat. These differences are also reflected in an increased sensitivity of the rabbit to benzene-induced suppression of peripheral lymphocyte numbers (Table 2, Fig. 9). Normal rabbit marrow exhibits about twice the proportion of cells in S as the rat, consistent with the indication that benzene affects an S phase-population of cells. Based on this evidence we are currently testing the hypothesis that sensitivity to benzene may be modulated by conditions that promote cycling and partial synchrony in proliferating bone marrow cell populations.

Flow cytofluorometric DNA cell cycle analysis makes possible the identification of early proliferative alterations in blood precursor cells in bone marrow often in the absence of change in peripheral blood cell parameters. Cycle or phase specific changes in proliferating cells may reveal clues as to the mechanism of toxic insult and can be used to discriminate b ——— ents with markedly different myelotoxic effects.

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