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THE SENSITIVITY OF HUMAN BONE MARROW GRANULOCYTE/MONOCYTE PRECURSOR CELLS TO PHENYLBUTAZONE, OXYPHENBUTAZONE AND GAMMA-HYDROXYPHENYLBUTAZONE *IN VITRO*, WITH OBSERVATIONS ON THE BONE MARROW COLONY FORMATION IN PHENYLBUTAZONE-INDUCED GRANULOCYTOPOENIA

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Abstract—The inhibitory actions of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone on the growth of human bone marrow granulocyte/monocyte colonies *in vitro* have been compared. Oxyphenbutazone is more toxic than phenylbutazone in this system. γ-hydroxyphenylbutazone is inhibitory over a wider concentration-range than either phenylbutazone or oxyphenbutazone, but has about the same inhibitory potency as phenylbutazone at concentrations corresponding to the peak plasma concentrations during treatment. Three patients who had recovered from phenylbutazone-induced bone marrow depression with neutropoenia were studied. The proliferative capacity of the bone marrow from all three patients was reduced as judged by the measured formation of granulocyte/monocyte colonies *in vitro*. The sensitivity of the patients' bone marrow to inhibition by either phenylbutazone (two patients) or by oxyphenbutazone (three patients) was compared with that of normal subjects' bone marrow. Increased sensitivity to phenylbutazone was demonstrated in the patients. The difference between the patients' and normal subjects' marrow with respect to their sensitivity to oxyphenbutazone was insignificant. The present results are compared with the results of similar studies with other drugs which produce neutropoenia. It is concluded that they are not inconsistent with the suggestion that phenylbutazone-induced neutropoenia is due to an underlying marrow abnormality.

Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidine-dione) is widely used in rheumatological practice, and agranulocytosis, aplastic anaemia and thrombocytopoenia are well recognised adverse side effects. The drug is metabolised to oxyphenbutazone (4-butyl-1,2-(4′-hydroxyphenyl)-3,5-pyrazolidine-dione) and γ-hydroxyphenylbutazone (4-(3′-hydroxybutyl)-1,2-diphenyl-3,5-pyrazolidine dione) *in vivo*. Oxyphenbutazone is also used therapeutically, and can depress the bone marrow [1]. The overall toxicity of phenylbutazone is presumably due to both that of the parent compound and its metabolites.

Bone marrow cells proliferate and differentiate into granulocytes and monocytes in semi solid agar provided that a source of the specific glycoprotein colony stimulating factor is present [2]. This phenomenon can be made the basis for an assay of either colony stimulating factor or of the proliferative activity of bone marrow and its sensitivity to myelotoxic drugs [3]. Greenberg and Schrier [4] reviewed some applications of *in vitro* bone marrow culture to the study of neutropoenia and myeloproliferative disorders generally.

The present studies were designed to: (i) compare the toxicities of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone towards human bone marrow granulocyte/monocyte precursor cells *in vitro*; (ii) determine if the bone marrow of patients, who had recovered from an episode of granulocytopoenia or agranulocytosis which had been attributed to phenylbutazone, could proliferate normally as judged by the formation of granulocyte/monocyte colonies *in vitro*, and if these cells were abnormally sensitive or abnormally resistant to either phenylbutazone or oxyphenbutazone.

MATERIALS AND METHODS

These studies were approved by the Ethical Committee of Northwick Park Hospital and Clinical Research Centre. The patients gave their informed consent to a sternal puncture and aspiration of marrow for the purpose of the investigations. Control human bone marrow was also obtained from ribs removed at thoracotomy for benign or malignant conditions and by sternal puncture from one of the investigators (RWEW) and a healthy professional colleague, who also gave his informed consent. All the specimens of control bone marrow were examined microscopically and judged to be normal by the usual morphological haematological criteria. Sternal bone marrow aspirates (0.5–2 ml) were transferred to conical centrifuge tubes after the addition of 2–3 drops of heparin (1000 i.u. ml^{-1} preservative free) and allowed to settle under gravity. The supernatant enriched in white cell precursors, was removed and centrifuged (10 min, $300\,g_{A_v}$). The cells were washed twice using 'Liebovitz' L15 medium (Flow Labora-

tories, Irvine, Scotland). Bone marrow cells were flushed from the rib specimens using: 'Liebovitz' L15 medium containing 2–3 drops heparin (1000 i.u. ml^{-1}, preservative free). The cells were dispersed to form a single cell suspension by forcing the suspension through a 10 μm needle. This cell suspension was then treated in the same way as the leukocyte precursor enriched bone marrow preparation. The cell cultures were set up as described previously.

The drugs were dissolved in double strength modified Eagle's minimum essential medium [3] without added foetal bovine serum, complete solution of these relatively insoluble compounds was only achieved by raising the pH value to about 10. The pH value was then readjusted to 7.4, the foetal bovine serum added (final concentration 18% v/v) and the vol. adjusted with glass-distilled deionised water to give single strength modified Eagle's minimum essential medium [3]. The final concentrations of these drug-solutions was 1.25 mM. This corresponds to a concentration of 250 μM in the culture dish. The concentrated (1.25 mM) drug solutions were diluted to give the desired final concentrations by adding single strength modified Eagle's minimum essential medium [3]. Portions (0.5 ml) of these diluted drug solutions or control drug-free medium were pipetted over the semi solid agar layer containing the bone marrow cells. Most of the observations were performed in quadruplicate, and the same batch of foetal bovine serum (Flow Laboratories, Irvine, Scotland) was used throughout to permit the assessment of proliferative capacity of the bone marrow granulocyte/monocyte precursors.

In the studies to compare the inhibitory effects of the three compounds on normal bone marrow cells, the groups of cells in the whole dish were counted after 10 days incubation as described previously [3]. Groups containing between 4 and 49 cells were scored as aggregates and those containing more than 49 cells were scored as colonies. Additional observations were made in the studies of the proliferative capacity of the patients and the relevant normal control subjects. Here, the groups of cells present in a randomly selected half of each culture dish were counted at 2 day intervals during the culture period. This modification in the area of the dish counted was introduced in order to reduce the period of time for which the culture dishes were exposed to the ambient atmosphere during the counting process, and because of the large number of dishes which had to be counted in these particular experiments. The cells were counted in the warm room (37°) in which they were incubated. The patients' and the control subjects' cells were treated identically.

Dose–response curves relating the drug concentration to the number of colonies were obtained and analysed by a probit method as described previously [3]. Percentage inhibition of colony formation was calculated after estimating the mean number of colonies formed in the absence of drug. As the present data are consistent with the number of colonies formed at a fixed drug concentration having a Poisson distribution the tests for parallelism and coincidence of the regressions of normal equivalent deviate on logarithm of drug concentration are made by goodness of fit tests. The data described previously [3] showed more variability than was expected from the assumption of the Poisson distribution, and were handled accordingly.

PATIENTS

Patient 1. This woman took phenylbutazone (300 mg daily) for 4 wk in February 1970 and for about 6 wk in May–June 1970 when she was 61 yr old, the drug being prescribed because of sacro-iliac pain. She developed purpura and ecchymoses at the end of the second period of phenylbutazone administration, and proved to have aplastic anaemia (haemoglobin 70%, platelets 15000/μl, total leukocyte count 800/μl with increased bleeding and clotting times). She is known to have had a normal haemoglobin (90%) and a morphologically normal stained blood film immediately before the first course of phenylbutazone. Treatment with blood transfusions and prednisolone produced a satisfactory clinical improvement although she has remained moderately anaemic. There is no history of other drug medication although the patient had received deep X-ray therapy after an ovarian cystectomy in 1957. The present studies were undertaken in 1975 when the patient was well clinically. The haematological data at this time are summarised in Table 1.

Patient 2. This woman, who has rheumatoid arthritis, was treated with phenylbutazone (300 mg daily) for 8 wk in 1967 when she was 41 yr old. She took calcium aspirin (3.6 g daily) concurrently with the phenylbutazone, having taken a smaller dose of calcium aspirin during the previous 4 months. The patient developed a sore throat and an ear infection, which did not respond to penicillin treatment, and she proved to be markedly neutropoenic (total white blood count = 1,400/μl, neutrophils 84/μl) having been normal (total white cell count = 7,600/μl) before phenylbutazone was given. The sternal bone marrow was relatively acellular with abnormal primitive cells of reticulum cell type and abnormal megakaryocytes; the numbers of erythroid and myeloid cells were markedly reduced, the picture suggesting an acute maturation arrest. She was treated with penicillin, prednisolone and nystatin, and improved so that one month after the onset of the acute episode her total white blood cell count = 7,200/μl, neutrophils = 5,190/μl, and the bone marrow was normal. The present studies were undertaken in 1975, when the patient was asymptotic and the haematological findings are summarised in Table 1.

Patient 3. This patient developed granulocytopoenia, thrombocytopoenia and anaemia in 1970 when she was 71 yr old, and whilst she was taking phenylbutazone (200–300 mg daily) having taken the drug intermittently in this dosage for the previous five yr. She was treated with corticosteroids, and antibiotics, and gradually recovered over the course of the following yr. Since when, she has been well. The present studies were undertaken in 1975 when the patient was asymptomatic and the haematological findings are summarised in Table 1.

RESULTS

The effects of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone on the proliferation of normal

Table 1. Haematological data when the patients were studied

	Patient 1 (M.P.)	*Patient 2* (E.R.)	*Patient 3* (E.H.)
Haemoglobin (g/100 ml)	12.2	14.1	13.1
Erythrocytes ($\times 10^6/\mu l$)	3.73	4.97	4.34
Packed cell vol. (%)	34.2	41.9	39.2
Mean corpuscular vol. (fl)	91	84	90
Mean corpuscular haemoglobin (pg)	33.2	28.2	30.3
Mean corpuscular haemoglobin concentration (%)	36.0	33.7	33.4
Total leukocyte count ($/\mu l$)	3600	5800	3900
Neutrophils ($/\mu l$)	2196	4176	1170
Lymphocytes ($/\mu l$)	1224	392	2418
Monocytes ($/\mu l$)	180	116	312
Eosinophils ($/\mu l$)	0	58	0
Basophils ($/\mu l$)	0	58	0
Reticulocyte count (%)	3	0.4	1.8
Platelets ($10^3/\mu l$)	155	370	195
Morphology of stained marrow	Cellularity normal. Iron –. Normoblastic erythropoiesis. Myeloid nuclei unusually small and regular. Megakaryocytes present. No other cells present. Comment: No diagnostic features.	Cellularity normal. Iron present. Normoblastic erythropoiesis. Normal granulopoeisis. Megakaryocytes present. No other cells present. Comment: No diagnostic features	Cellularity and iron content increased. Erythropoiesis very active, normoblastic with many early erythroblasts. Many early myeloid precursors, with reduced numbers of metamyelocytes and mature granulocytes. Normal granulopoietic morphology. Megakaryocytes present. Clusters of plasma cells, numerous reticulum cells and macrophages. Comment: Hyperplastic marrow with paucity of mature myeloid cells.

Table 2. The effect of phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone on control human bone marrow cells and of phenylbutazone and oxyphenbutazone on bone marrow cells from patients who had experienced phenylbutazone-induced bone marrow depression

Drug	Subject (Bone marrow identification)	Combined slope for group	ED_{50} (95% fiducial limits) (μM)	95% Range
Phenylbutazone	Control P6	10.09 ± 1.05	188.6 (171.4–202.6)	120.6–295.0
	Control P11		148.8 (136.8–159.4)	95.1–232.7
	Control P13		175.2 (161.3–188.4)	112.0–274.0
	Control P14		170.8 (159.1–181.7)	109.2–267.2
	Patient 1		101.0 (89.0–113.0)	64.6–157.9
	Patient 3		139.2 (119.7–160.2)	89.0–217.8
Oxyphenbutazone	Control P12	5.36 ± 0.30	24.2 (20.4–28.5)	10.4–56.1
	Control P19		40.6 (34.4–47.5)	17.5–94.2
	Control P24		31.0 (27.2–34.9)	13.4–71.9
	Control P27		29.2 (26.6–31.8)	12.6–67.8
	Control P30		42.5 (38.1–47.0)	18.3–98.6
	Control P35		44.9 (40.2–49.9)	19.4–104.2
	Control P37		33.1 (30.2–36.1)	14.3–76.9
	Patient 1		23.7 (19.9–27.9)	10.2–55.0
	Patient 2		28.6 (24.6–33.1)	12.3–66.5
	Patient 3		28.8 (19.6–42.4)	12.4–66.9
γ-Hydroxyphenylbutazone	Control P22	2.27 ± 0.31	271.6 (154.2–292.7)	28.9–1550.5
	Control P28		218.3 (167.4–283.7)	29.8–1600.0
	Control P33		152.3 (102.5–220.0)	20.8–1116.3
	Control P38		182.0 (135.5–238.8)	24.8–1333.4
	Control P39		154.3 (113.8–197.0)	21.1–1130.5

The slopes refer to the linear regression equations of normal equivalent deviate (NED) on the logarithm of drug concentration. The values given are the combined slopes for all of the experiments with one drug because they were parallel (see text). The ED_{50} values are the drug concentrations, which produced 50 per cent inhibition of colony formation.

human bone marrow to form granulocyte/monocyte colonies in vitro. The numerical results of these studies are shown in detail in Table 2.

The regression lines of normal equivalent deviates of inhibition on logarithm of the drug concentrations were all parallel for the within drug comparisons. The chi-squared test for parallelism of the dose–response curves from the normal marrows for phenylbutazone gave $\chi^2_3 = 1.42$ ($0.7 < P < 0.75$), for oxyphenbutazone $\chi^2_6 = 6.98$ ($0.3 < P < 0.5$) and for γ-hydroxyphenylbutazone $\chi^2_4 = 7.24$ ($0.1 < P < 0.2$). Those for phenylbutazone and γ-hydroxyphenylbutazone were also coincident in the within drug comparisons.

The regression lines for oxyphenbutazone were not coincident ($\chi^2_6 = 75.99$; $P < 0.001$) although they were parallel.

The ED_{50} values (drug concentration at which there is 50% inhibition of colony formation) are in the order: oxyphenbutazone < phenylbutazone < γ-hydroxyphenylbutazone. Thus, oxyphenbutazone is the most toxic of the three compounds from the standpoint of inhibiting granulocyte/monocyte colony formation. However, the slope of the regression line for γ-hydroxyphenylbutazone is low so that it produces some inhibitory effect over a wider range of concentration than the other compounds and is more toxic than phenylbutazone at lower concentrations. The curves cross in the region of the peak concentrations which are observed during phenylbutazone-treatment (197–487 μM) [5]. This is shown diagrammatically in Fig. 1. The corresponding data for phenylbutazone and oxyphenbutazone obtained with bone marrow from the patients are also shown in Fig. 1.

The ability of the bone marrow from patients who have had bone marrow depression associated with phenylbutazone treatment to proliferate in vitro. The reduced proliferative capacity of the patients' bone marrow *in vitro* is shown in Figs. 2 and 3. The results presented in Fig. 3 show that the low colony counts observed after 10 days of culture were not due to an increase in the ratio of cell aggregates (groups of 4–49 cells) to colonies (groups of >49 cells).

The effects of phenylbutazone and oxyphenbutazone on the proliferation of bone marrow from patients who have had bone marrow depression associated with phenylbutazone therapy. Phenylbutazone. The slopes (weighted regression coefficients of normal equivalent deviates on log dose) for the two patients were not significantly different from the controls ($\chi^2_5 = 1.44$, $0.9 < P < 0.95$; test for parallelism of all 6 curves). Using the combined slope in calculating the ED_{50} values (Table 2) these values for the patients were significantly lower than those of the control curves ($t_4 = 2.98$; $P = <0.05$). Thus, phenylbutazone appears to act in the same way in the patients' marrow and in the control subjects' although the patients' marrow is more sensitive to the inhibitory action of the drug.

Oxyphenbutazone. The curves obtained for the three patients were parallel with one another and their slopes were not significantly different from the controls ($\chi^2_9 = 7.52$; $0.5 < P < 0.7$; test for parallelism of

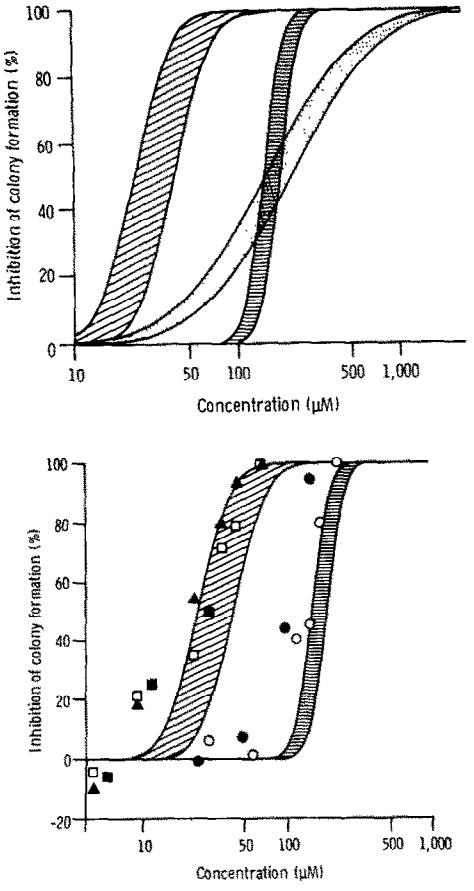

Fig. 1. The range of the control dose–response curves found for phenylbutazone, ▤; oxyphenbutazone, ▨; and for γ-hydroxyphenylbutazone, □. In the lower panel, the results obtained for the inhibition of colony formation in three patients by phenylbutazone (● patient 1; ○ patient 3) and by oxyphenbutazone (▲ patient 1; □ patient 2; ■ patient 3) have been superimposed on the ranges of the corresponding control data.

all 10 curves). The ED_{50} values for the patients were not significantly less than those of the controls by the *t*-test ($t_8 = 1.71$). Patient 1 had the lowest ED_{50} for oxyphenbutazone as well as for phenylbutazone (Table 2).

DISCUSSION

The present results show that phenylbutazone and its two metabolites are directly toxic to human granulocyte/monocyte progenitor cells *in vitro*. The extent to which each compound contributes to the depression of bone marrow function, and of granulopoiesis in particular *in vivo* is not known. The other known phenylbutazone metabolite, γ-hydroxyphenbutazone (4-(3′-hydroxybutyl)-1,2-(4′-hydroxyphenyl)-3,5-pyrazolidine-dione) was not available for study. *In vivo* toxicity depends on several pharmacokinetic factors which include: the extent of protein-binding, the rate of excretion and the rates of metabolism of the administered drug to different metabolites with different relative toxicities. Protein binding also has to be considered in *in vitro* systems, which contain serum, as in the present case.

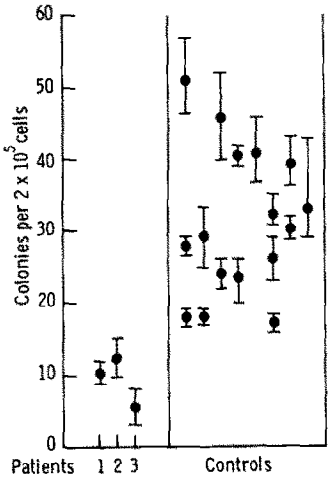

Fig. 2. Bone marrow colony formation in three patients who had recovered from phenylbutazone-induced bone marrow depression and 16 control subjects. In most cases, each point is the mean of 4 cultures from the same specimen of marrow. The bars show the extreme range of values upon which each mean is based.

Whittaker and Evans [6] concluded that phenylbutazone metabolism is controlled by both polygenic and environmental factors, the genetic contribution being about 66 per cent. Cunningham *et al.* [7] presented evidence suggesting that reduced oxidation of phenylbutazone to oxyphenbutazone might be a factor associated with, or responsible for, drug-associated bone marrow hypoplasia. In so far as the present *in vitro* observations can be extrapolated to the *in vivo* situation, they do not support this contention, because phenylbutazone is less toxic than oxyphenbutazone ($(ED_{50}$-oxyphenbutazone$) < (ED_{50}$-phenylbutazone)). However, this difference could be offset *in vivo* by the pharmacokinetic factors. γ-hydroxyphenylbutazone has a similar ED_{50} to phenylbutazone, with

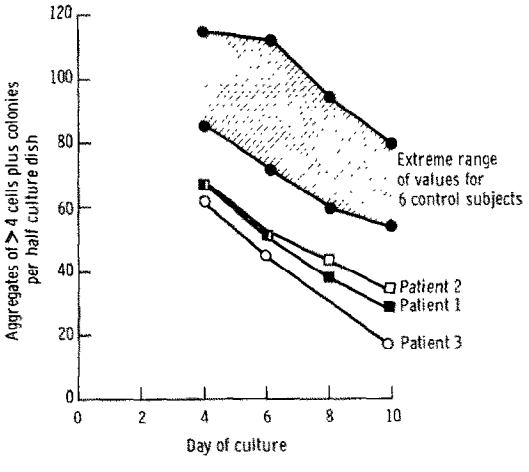

Fig. 3. The number of cell aggregates (groups of 4–49 cells) plus colonies (groups of >49 cells) per half culture dish when the bone marrow cells were grown without any of the drugs and counted at the 2 day intervals shown. The shaded area represents the extreme range of values for 6 specimens of normal marrow including those which were grown simultaneously with the patients marrow samples.

some effect over a wider concentration range so that it could contribute appreciably to the overall toxicity of phenylbutazone. Bakke *et al.* [8] found that the side chain oxidised metabolites including γ-hydroxyphenylbutazone are excreted more rapidly than phenylbutazone or oxyphenbutazone.

The concentration ranges which were studied in order to determine the ED_{50} values were established in preliminary experiments over a wide range of concentrations. In the case of phenylbutazone they cover the reported range of peak concentrations observed during therapy [5, 9]. It is of interest that, among the enzymes which catalyse the first five steps on the pathway of pyrimidine biosynthesis *de novo*, dihydroorotate dehydrogenase (EC 1.3.3.1) is uniquely sensitive to phenylbutazone inhibition in this concentration range [9]. Data on the plasma concentrations of oxyphenbutazone and γ-hydroxyphenylbutazone during phenylbutazone therapy are not available. However, plasma concentrations of drugs may be a poor guide to their concentrations in the target organs or at the active sites on drug-sensitive enzyme molecules. There is a discrepancy between the present finding of inhibition of granulocyte/monocyte precursor proliferation by phenylbutazone with an ED_{50} of about 180 μM and the previous report [3] that there was no inhibition in the range 5–500 μM. We ascribe this discrepancy to failure to completely dissolve the highest concentration of phenylbutazone in the Tris buffer which was used in the earlier work. Later experiments showed that the technique described under Methods is necessary in order to ensure that high concentrations of phenylbutazone remain in solution. The present results show that the drug has a direct toxic effect on the marrow and are in better accord with the suggestion that phenylbutazone-induced bone marrow depression is due to a direct myelotoxic action, which may reflect genetically determined differences in the mechanisms by which different individuals metabolise the drug [6].

Phenylbutazone inhibits mitosis in human lymphocytes [10], and the present finding that oxyphenbutazone in man is more toxic towards growing bone marrow cells than phenylbutazone, agrees with the observation that oxyphenbutazone depressed DNA synthesis in bone marrow cells more powerfully than phenylbutazone [11].

The proliferative capacity of the patients' bone marrow was reduced, and the granulocyte/monocyte precursor cells were more sensitive to the inhibitory properties of phenylbutazone than control subjects' marrow. These observations contrast sharply with the results of previous similar studies with chloramphenicol [12] and with sodium aurothiomalate [13]. The proliferative capacity of the marrow was reduced in two cases of delayed chloramphenicol-induced bone marrow depression, but the cells were more resistant to chloramphenicol *in vitro* than were control cells [12]. In the case of sodium aurothiomalate, although the bone marrow proliferative capacity was reduced during the acute phase, it was normal after recovery from the neutropoenia and was not abnormally sensitive to the inhibitory action of the drug *in vitro* [13]. These differences may reflect different mechanisms whereby drugs cause neutropoenia as an adverse side effect. The results of bone marrow culture studies in sodium aurothiomalate-induced neutropoenia were interpreted as indicating an alteration in the patients' overall metabolism of the drug, which causes high local concentrations in the bone marrow and a direct myelotoxic action [13]. The results with phenylbutazone are compatible with an underlying bone marrow abnormality rather than abnormal systemic handling of the drug, but with no tendency to breed out lines of drug-resistant bone marrow cells during recovery.

Rickard, Brown & Kronenburg [14] showed that an agar colony system similar to that used in the present work assays the committed myeloid stem cell compartment of human bone marrow. The present results show that it is possible to use *in vitro* bone marrow culture as the basis of a test system in which the inhibitory potency of potentially myelotoxic agents can be assessed as well as to quantitate the proliferative potential of a patient's bone marrow. Further refinements of the technique might make it useful as an adjunct in the testing of new drugs.

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