THE EFFECTS OF DRUGS THAT CAUSE NEUTROPENIA UPON COLONY FORMATION BY BONE MARROW CELLS IN SEMI-SOLID AGAR

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SUMMARY

1. The effect of drugs that cause neutropenia has been tested upon normal bone marrow colony formation in semi-solid agar.
2. Thiouracil, methimazole and phenylbutazone do not cause inhibition whereas sodium aurothiomalate, 6-mercaptopurine and chloramphenicol cause dose-related inhibition of colony formation at or below drug concentrations found in serum during therapy. Chloramphenicol also inhibits mouse bone marrow, L cells and Hela cells in agar culture.
3. Dose–response curves have been analysed by the probit method and found to be reproducible.

Key words: drug-induced neutropenia, bone marrow culture, colony formation.

Bone marrow precursor cells will proliferate and differentiate to form colonies of granulocytes and monocytes in semi-solid agar under suitable conditions. A source of a specific glycoprotein called ‘colony-stimulating factor’ is necessary in the culture system for colony formation to occur (Bradley & Metcalfe, 1966).

Colony-stimulating factor is found in human urine and serum (Chan, Metcalf & Stanley, 1971; Robinson, Stanley & Metcalf, 1969), in spleen-conditioned culture medium (Paran, 1972) and in peripheral blood leucocytes (Pike & Robinson, 1970). It can also be extracted from a variety of mouse tissues (Bradley, Stanley & Sumner, 1971) or produced as a mouse tissue-conditioned medium (Bradley & Sumner, 1968).

Although the mode of action of many drugs that cause neutropenia is known, e.g. 6-mercaptopurine and amidopyrine, there are many others where the mechanism of toxicity is unclear. With normal bone marrow in vitro, drugs that have a direct toxic effect upon cells would be expected to inhibit proliferation of normal cells, whereas those that may act by immunological

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or idiosyncratic mechanisms would have no effect. We have tested this proposition with six drugs which can cause neutropenia \textit{in vivo}.

There is some evidence that chloramphenicol depresses the \[^{14}\text{C}]\text{formate\ incorporation\ into\ DNA\ and\ RNA\ of\ bone\ marrow\ cells\ taken\ from\ patients\ who\ have\ recovered\ from\ chloramphenicol-induced\ aplastic\ anaemia\ to\ a\ greater\ degree\ than\ in\ control\ bone\ marrow\ (Yunis,\ 1969).\ This\ suggests\ that\ the\ cells\ are\ abnormally\ sensitive\ to\ this\ drug.\ We\ have\ developed\ a\ reproducible\ bioassay\ for\ the\ effect\ of\ chloramphenicol\ upon\ normal\ bone\ marrow \textit{in vitro} by assessing the inhibition of colony formation in semi-solid agar. This allows the effect of chloramphenicol \textit{in vitro} on the bone marrow of patients who have recovered from neutropenia or aplastic anaemia to be observed.

Some of these results have been the subject of a preliminary communication (Howell, Andrews & Watts, 1973).

**METHODS**

**Cell culture**

Human bone marrow cells were cultured in semi-solid agar on a hard agar underlay by a modification of the technique of Pike & Robinson (1970). Aliquots (1 ml) of equal volumes of 1% agar and double-strength modified Eagle's medium, containing 18% foetal bovine serum (Bradley & Metcalfe, 1966), were pipetted into 30 mm plastic Petri dishes (Sterilin), together with \(10^6\) human peripheral blood leucocytes, as a source of colony-stimulating factor, and 0.1 ml of the test drug. Aliquots (1 ml) of equal volumes of 0.6% agar and double-strength Eagle's medium with \(2 \times 10^5\) normal human bone marrow cells were pipetted over the basal agar layer and allowed to gel at room temperature for 10 min.

The modified Eagle's medium used in these experiments contains: Eagle's minimum essential medium (M.E.M.) \((\times 10)\) (Flow Laboratories Ltd, Irvine, Scotland), 14.5 ml; Eagle's M.E.M. vitamins \((\times 100)\), 2.9 ml; Eagle's M.E.M. amino acids \((\times 100)\), 2.9 ml; Eagle's M.E.M. non-essential amino acids \((\times 100)\), 1.45 ml; sodium pyruvate \((0.2\,\text{mol}\,\text{l}^{-1})\), 1.45 ml; asparagine \((3.7 \times 10^{-2}\,\text{mol}\,\text{l}^{-1})\), 0.8 ml; serine \((9.5 \times 10^{-2}\,\text{mol}\,\text{l}^{-1})\), 0.2 ml; sodium bicarbonate \((0.72\,\text{mol}\,\text{l}^{-1})\), 8.2 ml; distilled water, 11 ml; glutamine \((0.2\,\text{mol}\,\text{l}^{-1})\), 2 ml; Trypticase Soy Broth (Baltimore Biological Laboratory) \((30\,\text{g}\,\text{l}^{-1})\), 18 ml; foetal bovine serum (Flow), 36 ml; benzylpenicillin \((10^8\,\text{units}\,\text{l}^{-1})\) and streptomycin \((0.2\,\text{mol}\,\text{l}^{-1})\), 0.2 ml.

Peripheral blood was collected into syringes treated with preservative-free heparin \((1:1000)\). The blood was allowed to sediment in the syringe under gravity for up to 2 h, the supernatant plasma and leucocytes were removed and washed twice in Liebovitz L15 tissue culture medium (Flow) before counting and then mixing with agar and medium for the under layers. Bone marrow cells, obtained either by sternal aspiration from normal volunteers (A.H., T.M.A.) or from ribs removed at thoracotomy, were suspended in heparinized foetal bovine serum and allowed to sediment for up to 2 h before removing the supernatant plasma and cells. These cells were washed twice in L15 medium before mixing with the agar medium for the upper layer.

Drugs were made up as 10 mmol l\(^{-1}\) solutions in phosphate-buffered saline, pH 7.4, except for phenylbutazone which was made up in Tris buffer, pH 8.4, and the pH adjusted to 7.6 by adding 2 mol l\(^{-1}\) HCl. Dilutions \((0.1\,\text{ml} \times 10^8\,\text{mmol}\,\text{l}^{-1}\) to \(0.02\,\text{mmol}\,\text{l}^{-1}\)) of each drug were added to the under layer. Phosphate-buffered saline was used in the control cultures except in the
experiments with phenylbutazone when Tris buffer (pH 7.6; 0.1 ml) was employed. All dilutions and controls were tested at least in triplicate.

The effect of chloramphenicol on murine bone marrow cells, murine L cells (Flow) and human Hela cells (Difco) growing in the agar culture system was also investigated.

Mice, 2–3 months old (CBA/Ca inbred strain, maintained at the Clinical Research Centre), were killed by cervical dislocation and the bone marrow was flushed from the femora with L15 medium. The cells were washed twice in L15 medium before counting and adding to the upper agar layer at a final concentration of $2 \times 10^5$ cells/ml. An extract (0.1 ml) of pregnant mouse uterus prepared according to the method of Bradley et al. (1971) was used as a source of colony-stimulating factor. L cells and Hela cells form colonies in agar without added colony-stimulating factor. One hundred cells were added to each Petri dish. The plating efficiencies were 28% and 35% for L cells and Hela cells respectively.

All cell types were incubated at 37°C in sealed and humidified plastic boxes containing 10% CO₂ in air. The colonies were counted with an inverted microscope when the human marrow cells had been incubated for 14 days and all other cell types for 7 days. A group of more than fifty cells was counted as a colony for bone marrow cells, and a group of more than ten cells was counted as a colony for the L cell and Hela cell cultures. Cell morphology was assessed by removing colonies from the agar with haematocrit tubes and staining on slides with aceto-orcein. Human colonies contained predominantly granulocytes or a mixture of granulocytes, monocytes and their precursors. Murine colonies were predominantly macrophages with occasional mixed and purely granulocyte colonies.

Statistical analysis

Percentage inhibition of colony formation was defined as

$$\frac{\text{estimated no. of initial colonies} - \text{no. of final colonies}}{\text{estimated no. of initial colonies}} \times 100$$

The percentage inhibition of colony formation is related to log concentration of drug by a sigmoid dose–response curve, with inhibition ranging from 0 to 100%. The usual method of fitting a curve of this type involves transforming the percentage inhibition to a ‘probit’ or normal equivalent deviate (NED) and performing a weighted regression of NED on log concentration (Finney, 1971). In our experiments the percentages of inhibition were not known precisely, as the denominator, the number of colonies formed in the absence of drug, is variable. Several controls were used in each experiment; it would be possible to arrange the data as above by expressing response as a percentage of the mean of the controls. A more precise analysis is made by including the mean of the number of colonies formed in the absence of drug as a parameter of the fitted curve, which has to be estimated at the same time as the parameters of the regression line, using methods developed by Wadley (1949) and Finney (1971).

Comparisons of toxicities of drugs, and in this case sensitivities of cells, are simplest when the NED regression lines corresponding to the dose–response curves are parallel. Parallelism of the regression lines for a group of experiments was tested by comparing two $\chi^2$ values for goodness of fit, the first obtained by fitting independent regression lines as above, the second by fitting separate but parallel lines to all sets of data. If a dose–response curve is reproducible, regression lines obtained from experiments using the same drug and type of cell should be
coincident. The $\chi^2$ value obtained from the parallel lines was compared with that found by fitting one regression line for all similar experiments. Control values depended on the culture conditions; the mean number of colonies formed in the absence of drug was estimated for each experiment.

The analysis was carried out using the mean number of colonies formed for each concentration of drug; the value of $\chi^2$ obtained was compared with a second value calculated from the individual observations to test for departures from the underlying model (Finney, 1971).

RESULTS

The observed effects of phenylbutazone, methimazole, thiouracil, chloramphenicol, 6-mercaptopurine and sodium aurothiomalate upon human bone marrow colony formation are shown in Fig. 1. The first three drugs have little or no effect whereas there is marked depression of colony formation with chloramphenicol, 6-mercaptopurine and sodium aurothiomalate at or below concentrations found in serum during therapy.

The effect of chloramphenicol was investigated further with a wider range of concentrations, and by testing the effect of this drug upon murine bone marrow, L cells and Hela cells. Dose-dependent inhibition of colony formation occurred with all cell types. A sigmoid dose–response

![Graphs showing effect of drugs on colony formation](image)

Fig. 1. Effect of six drugs upon human bone marrow colony formation in vitro, expressed as a percentage of the number of colonies in control Petri dishes.
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curve was satisfactorily fitted to data from each of the experiments with chloramphenicol. Significant \( \chi^2 \) values indicated heterogeneity of the test material, but no systematic departures from the linear regression line were found. Heterogeneity was allowed for in calculating standard errors. A dose–response curve showing the effect of chloramphenicol on human marrow cells is shown in Fig. 2, with therapeutic plasma concentrations shaded. The corresponding regression line of NED on log concentration is shown in Fig. 3, with its 95% fiducial limits.

![Graph](image_url)

**Fig. 2.** Relation between inhibition of colony formation, expressed as a percentage of estimated natural growth, and concentration of chloramphenicol. The therapeutic plasma concentration is shaded.

The estimates of the parameters of the individual dose–response curves are shown in Table 1 and the regression lines are shown graphically in Fig. 4. The slopes of the regression lines for the three experiments with mouse bone marrow cells were not significantly different \( (P = 0.17) \) and a single regression line did not show a significantly worse fit than the parallel lines \( (P = 0.27) \). Similarly one line was sufficient to describe the two experiments with human bone marrow cells, although the test for lack of coincidence gave a result which was almost significant at the 5% level.
Fig. 3. Relation between NED (normal equivalent deviate) of inhibition and concentration of chloramphenicol, showing regression line with fiducial limits (points with arrows represent 0% and 100% inhibition).

Table 1. Effect of chloramphenicol on human and murine marrow cells, Hela cells and L cells

The slopes and intercepts refer to the linear regression equations of normal equivalent deviate (NED) on the logarithm of chloramphenicol concentration.

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Parameters of regression line</th>
<th>Mean no. of colonies in absence of drug</th>
<th>ED$_{50}$ ($\mu$mol l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Intercept</td>
<td></td>
</tr>
<tr>
<td>Human marrow (H1)</td>
<td>3.32±0.58</td>
<td>−6.80±1.33</td>
<td>44.9±2.7</td>
</tr>
<tr>
<td>marrow (H2)</td>
<td>1.81±0.34</td>
<td>−3.67±0.82</td>
<td>50.5±3.1</td>
</tr>
<tr>
<td>Hela (H3)</td>
<td>2.51±0.17</td>
<td>−5.33±0.42</td>
<td>59.6±1.6</td>
</tr>
<tr>
<td>Murine marrow (M1)</td>
<td>0.93±0.16</td>
<td>−0.88±0.36</td>
<td>43.5±5.9</td>
</tr>
<tr>
<td>marrow (M2)</td>
<td>1.37±0.26</td>
<td>−1.51±0.51</td>
<td>83.4±10.8</td>
</tr>
<tr>
<td>marrow (M3)</td>
<td>1.66±0.29</td>
<td>−2.41±0.60</td>
<td>83.8±7.5</td>
</tr>
<tr>
<td>L (M4)</td>
<td>1.58±0.30</td>
<td>−1.93±0.58</td>
<td>27.9±3.3</td>
</tr>
</tbody>
</table>
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**FIG. 4.** Regression lines of NED (normal equivalent deviates) inhibition on concentration of chloramphenicol for individual experiments (---); the mean regression lines for human cells (H) and mouse cells (M) are also shown (-----).

**FIG. 5.** Values of ED₅₀ for all experiments with chloramphenicol. Bars represent 95% fiducial limits.
A single regression line could also be fitted to all four sets of data from mouse cells including the experiment with L cells; the response of HeLa cells was not significantly different from human marrow cells. The mean line for human cells, and that for murine cells, are shown in Fig. 4. However, the slopes of the regression lines from the human marrow cell data were significantly greater than those for the murine cells ($P<0.01$).

The estimates of ED$_{50}$ (median effective dose producing 50% inhibition of growth) are shown in Fig. 5. When two regression lines are parallel the ratio of the ED$_{50}$ values is a measure of relative sensitivity of the cells (and comparison of the ED$_{50}$ values is equivalent to testing for coincidence of the regression lines). As the regression lines for human cells were not parallel to those for murine cells the relative sensitivity changes as the drug concentration increases. Fig. 4 shows that for lower concentrations of drug mouse cells are more sensitive to chloramphenicol than human cells, but that there is little difference at the higher concentrations.

**DISCUSSION**

Chloramphenicol inhibits colony formation by human bone marrow cells in vitro at concentrations found in serum during therapy (Goodman & Gilman, 1970). This inhibitory effect is not confined to human marrow nor to marrow cells only, since mouse bone marrow, mouse L cell and human HeLa cell proliferation is also reduced in culture. In short-term tissue culture experiments, inhibition of proliferation of bone marrow occurs only at very high chloramphenicol concentrations ($>300$ $\mu$mol L$^{-1}$) (Yunis & Harrington, 1960; Ward, 1966) whereas low doses of chloramphenicol inhibit protein synthesis in bone marrow (Djordjevic & Szybalski, 1960) and myeloma cell proliferation (Harman, Petergell & Sorenson, 1969) if the culture period is prolonged beyond 24 h as was the case in our experiments. Scott, Finegold, Belthin & Lawrence (1965) found that granulocyte depression occurred in patients treated for more than 14 days and if the plasma chloramphenicol concentrations were consistently above 75 $\mu$mol L$^{-1}$. Bone marrow culture in agar mimics this time-course and inhibition of proliferation occurs at similar drug concentrations.

Chloramphenicol primarily affects mammalian cells in rapidly dividing tissues. It depresses haemopoiesis and is immunosuppressive in vivo (Weisberger, Moore & Schoenberg, 1966) and in vitro (Ambrose & Coons, 1963). However, it does not inhibit beating rat heart cells in tissue culture (Kroon & Jansen, 1968) nor protein synthesis in non-dividing lymphocytes (Smith & Forbes, 1967). Mouse cells are more sensitive than human cells over most of the range of concentrations used in our experiments; this may be due to the difference in the generation times of mouse and human marrow or to other species differences. The generation time for mouse bone marrow is approximately 12–15 h (Bøyum & Breivik, 1973; Testa & Lord, 1973), whereas estimates of the generation time for human marrow vary from 24 h (Stryckmans Cronkite, Fache, Fliedner & Ramos, 1966) to 52 h (Cronkite & Vincent, 1969).

Two types of bone marrow depression occur in association with chloramphenicol treatment (Yunis, 1969). One is relatively common, dose-dependent, occurs during therapy, is reversible and thought to be caused by inhibition of mitochondrial protein synthesis (Martels, Maryan, Smith & Yunis, 1969). The other is rare, non-dose-dependent, usually occurs after a course of therapy, and may be secondary to a biochemical defect in the patient's cells (Yunis, 1969). Inhibition of colony formation in vitro by chloramphenicol correlates well with a dose-dependent type of marrow depression in vivo, but does not explain why a few patients develop...
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fatal marrow aplasia. If the latter were due to a biochemical cellular defect the dose–response curve for affected cells might be expected to be different from the dose–response curve for normal cells.

Serum concentrations of sodium aurothiomalate in patients with rheumatoid arthritis range between 5 and 40 μmol l⁻¹ in our laboratory, and colony formation in vitro is inhibited by drug concentrations within this range. Neutropenia secondary to chrysotherapy is uncommon, and on the basis of experiments reported elsewhere (Howell et al., 1973), we have suggested that for the marked inhibitory effect of sodium aurothiomalate in agar culture is due either to inhibition of release of colony-stimulating factor from leucocytes in the agar under layer or due to the drug binding with colony-stimulating factor and rendering it inactive.

As expected of a drug which interferes with purine synthesis 6-mercaptopurine inhibits colony formation in vitro at low concentrations, but 2-amino-6-mercaptopurine (6-thioguanine) is ten times less active under similar culture conditions (A. Howell & R. O. McKeran, unpublished observations). Bone marrow culture may therefore be a useful way of testing different drugs with similar therapeutic effects for potential myelotoxicity.

Phenylbutazone, methimazole and thiouracil do not affect colony formation in the therapeutic range. These drugs may cause neutropenia in vivo because of an immunological reaction (Moeschlin, 1956; Huguenin, Albou & Griguer, 1959); under these circumstances they would not be expected to inhibit proliferation in vitro except possibly in the affected patients' serum.

As with many bioassays, considerable errors occur in estimating the parameters of the dose–response curves, and it may be that the differences of effects of some drugs upon cells are too small to be demonstrated by assays of a practical size. Our evidence suggests, however, that detectable differences of response occur between mouse and human cells and between some related drugs with similar therapeutic effects, for example 6-mercaptopurine and 2-amino-6-mercaptopurine.

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REFERENCES


