Bone Marrow Culture *in vitro*

Current Status and Some Clinical Applications

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SUMMARY

The *in vitro* culture of bone marrow is an established method for the study of normal and abnormal haematopoiesis. In a semi-solid agar system, marrow progenitor cells (CFU,) of the granulocyte and macrophage lines will clone in the presence of specific colony-stimulating factors (CSF) and appear in the matrix as clusters (6-50 cells) or colonies (more than 50 cells). Growth is not normally obtained from peripheral blood unless this is first concentrated. To characterize the morphology of these cells in situ, a method has been developed in which the entire plate is fixed and mounted on a slide. After drying, this preparation is well suited to staining with a variety of biological dyes and furthermore provides a permanent and detailed record of the entire culture.

Analysis of 414 studies in a variety of clinical conditions forms the basis of this communication. In a 35-mm Petri dish normal marrow grows 30 colonies (SEM ± 2.49) and 39 clusters (SEM ± 3.59) at 12 days of culture resulting in a colony:cluster ratio of 1:1.3. The technique was found useful in our bone marrow transplantation programme where scanty growth in culture identified those donor grafts containing a paucity of stem cells, which is one explanation for the failure to obtain haematopoietic reconstitution. In preleukaemia only 2,75 colonies (SEM ± 0.85) and 15 clusters (SEM ± 4.24) were obtained.

In overt leukaemia, whether arising *ab initio* or following relapse, plating efficiency was similarly reduced to 5.2 colonies (SEM ± 1.34) although 64 clusters (SEM ± 12.54) were found resulting in a colony:cluster ratio between 1:5 and 1:12. With successful therapy this pattern returns to normal, but reappearance of disturbed growth or aberration of colony:cluster ratio signals a relapse or the presence of minimal residual disease, a pattern that may occur when peripheral blood and marrow are still morphologically normal. In the myeloproliferative syndrome CFU become demonstrable in the peripheral blood as the disease progresses, and metamorphosis to either the accelerated phase or to blast transformation results in changes similar to those found in leukemic marrow.

These studies illustrate the potential practical value of this technique. Further applications include the exploration of aetiological mechanisms in patients with neutropenia and bone marrow aplasia, or in elucidating humoral or cellular factors inhibiting growth in patients with tumours such as myeloma. *In vitro* culture systems have diagnostic potential in haematology, and further evaluation will define their role in current practice.


The mechanisms controlling the orderly proliferation and maturation of haematopoietic cells within the bone marrow are complex and they have proved difficult to analyse because, until recently, there was no practical way of examining the influence of the individual regulating factors. Liquid suspension cultures of marrow suffer from many of the same limitations as *in vivo* experiments in that relatively small segments of the whole process cannot be isolated for study. Appreciating the need for alternative analytical techniques, Pluznik and Sachs and Bradley and Metcalf extended the experience of virologists that transformed cells will grow *in vitro* by attempting to culture tumour cells using similar methods. The two groups tried independently to obtain clonal growth in agar using mouse leukaemia or AKR lymphoid leukaemia cells respectively, but both were initially unsuccessful. Further experiments were based on the technique described by Puck and Marcus in which feeder layers of various cells were employed to obtain colony formation. However, although growth did occur, analysis of the cell aggregates showed that these were composed of neutrophilic granulocytes and macrophages derived from the underlayer and not of tumour origin. Nevertheless, these and other studies established that a specific growth factor was necessary for colony formation and that this was supplied by cells from the underlayer.

Rapid progress has been made from these important initial observations. Thus, Pike and Robinson successfully applied the double-layer technique to the study of human bone marrow, while Chervenick and Boggs substituted methylcellulose for agar as the matrix to support cell growth. Subsequently, Axelrad *et al.* demonstrated that erythroid colony formation could be obtained in the presence of erythropoietin. Metcalf *et al.* have been prominent in much of the development of semi-solid agar systems including isolation and purification of colony-stimulating factors and the use of mitogen-stimulated lymphocytes to support the growth of eosinophils as well as megakaryocytes. Furthermore, it was observed that B and T lymphocytes and haematopoietic stromal cells could be successfully cultured in the system.
containing 2-mercapto-ethanol.22-25 These various achievements have been reviewed by Metcalf8 and it is clear that the basic technique may, with modifications, provide a practical approach to the analysis of the various physiological processes that govern cell growth, including the relationship between haematopoietic cells and supporting stroma.

The same approach has been used to explore functional differences that may be recognizable between normal and neoplastic cells. In the case of leukaemia it has been reported26,27 that the tumour cells may be distinguished from their normal counterparts in culture, and this important observation is presently being further evaluated. The most obvious deviation that samples of leukaemic marrow show from normal are alterations in plating efficiency and disturbances in colony: cluster ratio. The latter is presumed to reflect, at least in part, profound suppression of normal haematopoietic populations. Other explanations are that leukaemic progenitor cells are inherently unable to mature normally, or that their response to regulatory factors may be defective. A variety of other malignancies have been examined by in vitro culture of bone marrow and most of this material has recently been analysed and reviewed by Metcalf.8 It is evident that these methods provide a means for the examination of a host relationship with the neoplasm, a chance to define tumour kinetics and provide at least one way of testing cytotoxic regimens.

One of the major limitations of the agar culture system is the difficulty in defining morphological detail of constituent cells within the colonies or the clusters, and this difficulty is reflected in the variety of techniques used for their study.10,11 The latter extend from phase-contrast microscopy through a variety of supravital techniques to those which necessitate aspiration of the colony from the matrix for mounting and subsequent staining.10,11 None of these approaches is really practical for the study of cells in situ, nor do they offer a permanent record of the entire culture for later comparative studies. Furthermore, although described methods will allow a degree of morphological recognition, this is achieved at the cost of disrupting cellular relationships. We have recently developed a technique which makes it possible to realize these objectives.22

In recent years there has been a steadily increasing application of the in vitro culture of marrow for studying the physiological and pathological features of haematopoiesis, and improvements in methodology regularly appear in the literature. We have analysed our experiences with this assay system and report these in the context of clinical applications of the method.

MATERIAL AND METHODS

The 414 studies which were carried out are analysed in Table 1. Underlayers were prepared each week in 35-mm Petri dishes (Falcon 3001) containing $1 \times 10^5$ normal leucocytes/ml of 0.5% agar (Difco). Marrow or blood cells to be studied were harvested from dextran sedimentation and overlayered as a monocellular suspension in 0.3% agar at a concentration of $2 \times 10^5$ cells/ml. Petri dishes were incubated at 37°C in a humidified atmosphere containing a final concentration of 5% carbon dioxide. All studies were done in triplicate and included control cultures using NIH-conditioned medium derived from human embryonic kidney (J. M. Bull - personal communication). The plating efficiency was determined by counting the colonies and clusters on days 10, 12 and 14, using either an inverted microscope with phase-contrast illumination or preferably a stereo dissecting microscope with an incident light source. Parallel cultures were set up in agar without underlayer or additional conditioned medium to

### TABLE I. ANALYSIS OF 414 STUDIES CARRIED OUT

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number</th>
<th>Mean</th>
<th>Range</th>
<th>SEM</th>
<th>Mean</th>
<th>Range</th>
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<td>29.9</td>
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<td>39.1</td>
<td>16 - 46</td>
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<td>1 - 5</td>
<td>0.85</td>
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<td>50</td>
<td>5.2</td>
<td>0 - 220</td>
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<td>63.94</td>
<td>0 - 300</td>
<td>12.54</td>
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<td>12</td>
<td>8.17</td>
<td>0 - 28</td>
<td>2.59</td>
<td>23.83</td>
<td>8 - 46</td>
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<td>Acute non-lymphoblastic leukaemia in remission</td>
<td>62</td>
<td>30.87</td>
<td>0 - 81</td>
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<td>88</td>
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<td>0 - 305</td>
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<td>26.32</td>
<td>0 - 129</td>
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<td>17.61</td>
<td>0 - 50</td>
<td>2.12</td>
<td>34.08</td>
<td>4 - 107</td>
<td>4.19</td>
<td>1:1.9</td>
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<td>2.63</td>
<td>0 - 10</td>
<td>1.15</td>
<td>35.83</td>
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monitor the spontaneous growth rate and the colony: cluster ratio. Marrow cells from patients with myeloma were washed in tissue culture medium and then cultured in either autologous or AB serum to demonstrate the presence of humoral inhibitors to *in vitro* growth.

At the completion of the culture period, the mean number of colonies and clusters for each plate was determined and the ratio recorded. The entire Petri dish was then flooded with fixative; the agar discs were freed and transferred to a watch glass with excess fixative. After separation from the underlayer, which was discarded, the overlayer was mounted on a glass slide, air-dried and stained using a variety of biological dyes and cytochemical reactions. Morphological screening was done using Romanowsky, haematoxylin-eosin and methyl green-pyronin stains. When indicated, esterase, Sudan black B or myeloperoxidase reactions were employed. For ultrastructural studies, colonies were selected from plates that had been fixed in 3% glutaraldehyde (pH 7.2); these were processed and sectioned at 400 Å on an ultramicrotome. Grids were viewed on a Siemens electron microscope and appropriate fields were photographed.

**RESULTS**

The results of the 414 studies at the 12-day period of culture are seen in Table I and Fig. 1. From normal marrow a mean of 29.9 colonies (SEM ± 2.49) and 39.1 clusters (SEM ± 3.59) was found; the colony: cluster ratio is 1 : 1.3. As the culture ages, the morphological features of the cells change from neutrophils (Fig. 2) to a period when macrophages are also present, until finally only macrophages are found (Fig. 3).

Samples obtained from bone marrow transplant donors gave results in the normal range. However, in two cases a marked decrease in plating efficiency was found and neither of the recipients achieved engraftment despite the presence of adequate numbers of nucleated cells, the latter being the usually accepted criteria for graft adequacy.

In patients with preleukaemia there is a marked reduction in overall plating efficiency reflected in the colony count of 2.75 (SEM ± 0.85) and clusters of 15 (SEM ± 4.24). The colony: cluster ratio may initially be within the normal range but typically a disproportionate number of clusters grow, resulting in the abnormal ratio of 1 : 5.5.

In patients with acute non-lymphoblastic leukaemia, both plating efficiency and colony formation are markedly reduced. Colony formation was 5.2 (SEM ± 1.34), but abundant cluster formation, 63.94 (SEM ± 12.54), was seen.
resulting in profound disturbance in the colony: cluster ratio. A similar pattern was seen in acute lymphoblastic leukaemia, where subnormal colony and cluster formation was found (8.17 ± 2.59 and 23.83 ± 3.6 respectively). For the two morphological variants the ratios were 1 : 12.3 and 1 : 3 respectively. These deviations from normal are statistically significant ($P<0.05$).

Patients with adult acute leukaemia, whether non-lymphoblastic or lymphoblastic, who had achieved and maintained complete remission, are characterized by normal mean colony counts of 30.87 (SEM ± 2.85) and 22.43 (SEM ± 2.99) respectively. Similarly, cluster counts were 56.02 (SEM ± 3.86) and 47 (SEM ± 6.63) for the two leukaemic variants. Colony: cluster ratios for the two groups of leukaemic patients in remission were 1 : 1.8 and 1 : 2.1. None of the values deviates significantly from normal (0.04<$P<0.05$).

In one patient with acute non-lymphoblastic leukaemia, a transient marked increase in overall growth with abundant colonies preceded the more usual changes in ratio by 3 months (Fig. 4) and the patient subsequently relapsed. This pattern may be another manifestation of occult disease and should alert physicians to the possibility of impending deterioration. The change in growth pattern was associated with an alteration in the character of the colonies, which were large and loosely packed with cells, a feature that is quite distinct from the usual density encountered during remission.

In patients with the myeloproliferative syndrome the pattern did not initially differ significantly from the normal; no growth was obtained from blood while marrow yielded mean counts of 42.55 colonies (SEM ± 10.85) (0.7<$P<0.8$) and 46.3 clusters (SEM ± 8.94) (0.6<$P<0.7$). However, with progression of the disease, a marked contrast from normal peripheral blood emerged. Normally unconcentrated blood grows virtually no colonies or clusters, whereas in the myeloproliferative syndrome 26.32 colonies (SEM ± 5.54) and 41.47 clusters (SEM ± 8.38) were found. The onset of accelerated phase or blastic transformation was characterized by a decrease in plating efficiency in the blood and a prominent disturbance in colony: cluster ratio, a finding that closely parallels that obtained from leukaemic marrow.

In myeloma, colony formation is generally depressed to a level of 17.61 (SEM ± 2.12) while cluster counts are normal at 34.08 (SEM ± 4.19). It is noteworthy that no significant difference is demonstrable when washed marrow cells from patients with myeloma are cultured in either autologous or in AB serum, indicating that humoral inhibition of granulocyte: macrophage colony formation is unlikely to be found in the patient's serum.

In neutropenic subjects, colony: cluster ratio was not significantly different from normal, with values of 12.8 (SEM ± 2.87) and 35.83 (SEM ± 1.78) respectively. These observations indicate that peripherally acting mechanisms were present in our patients rather than production defects in the stem cells, where poor growth would have been anticipated.

In aplastic anaemia, colony formation was markedly depressed to 2.63 (SEM ± 1.15) with cluster counts of 23.38 (SEM ± 5.71), findings characteristic of a stem cell lesion. It was not, however, possible to exclude the possibility that these findings may reflect inhibition occurring on the basis of cell-to-cell interaction taking place in the culture.

**DISCUSSION**

The demonstration that progenitor cells (CFUc) of the granulocyte: macrophage lines derived from samples of bone marrow have the capacity to clone in vitro gave considerable impetus to the use of this technique for the study of both normal and abnormal haematopoiesis. Before the availability of semi-solid agar culture methods, the complex nature of the regulatory mechanisms operating within the intact bone marrow and the difficulty of isolating segments of the differentiation sequence made analytical studies almost impossible. Although liquid cultures were used, they had many of the same limitations that applied to the in vivo experiments. For example, the effects of any manipulation were seen only at the end of a chain of related events, and it was difficult to segregate and manipulate individual phenomena occurring during haematopoietic proliferation and maturation. In contrast, the suspension of progenitor cells in a viscous matrix makes it possible to explore some of these physiological processes and their regulation at cellular and humoral level.

The current technique has been developed from the early studies of Pluznik and Sachs¹ and Bradley and Metcalf,² with subsequent adaptation of the method to human material by Pike and Robinson.³ Further refinements have followed with the preparation of the colony-

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**Fig. 4. Serial studies in a patient with acute non-lymphoblastic leukaemia.** In early 1976 the colony: cluster ratio was normal. In the middle of 1976, when the patient was clinically and haematologically normal, a marked increase was documented in both colonies and clusters. Early in 1977 profound disturbance of the growth pattern was manifest and 6 months later, for the first time, haematological relapse occurred.
stimulating factor (CSF) from a variety of different tissue sources, and standardization can be achieved by titrating varying quantities of this material against fixed numbers of CFU from bone marrow that has been previously characterized and then stored. The regular use of such dose-response curves and the inclusion of an international standard (J. M. Bull — personal communication) will provide quality control and enable the results between laboratories to be compared.

In physiological terms the culture of bone marrow in agar has been instrumental in demonstrating that a relationship exists between the granulocyte and the macrophage: monocyte system, and this, in turn, has led to the enunciation of a hypothesis for the homeostatic regulation of granulocyte mass. Basically it appears that macrophages elaborate a CSF that promotes granulopoiesis and as the granulocyte pool expands it produces increasing quantities of a second humoral factor or chalone, having colony-inhibiting activity (CIA) which, in an adequate concentration, will suppress granulopoiesis. Since this is a dynamic system, a critical point will be reached where the balance between these two factors again alters, with the result that macrophage-derived CSF becomes dominant and CFU favours neutrophil production. It has also been suggested that bacteria may play a pivotal role in granulocyte production by serving as a stimulus to increase in CSF elaboration, and as the newly formed cells remove the antigen, the CSF level falls and the neutrophil generation decreases. More recently it has been shown that a similar negative feedback loop may be mediated by macrophage-derived prostaglandin E production. From these studies it is evident that in vitro bone marrow culture has an important role to play in elucidating the mechanisms that interact to control granulopoiesis.

In bone marrow transplantation graft rejection occurs in a number of patients, and this is usually on the basis of prior exposure to blood products with sensitization and iso-antibody formation. Alternatively, it is possible that insufficient numbers of stem cells may be infused and, here also, engraftment will not be achieved. It is a simple matter to culture a sample of the donor graft and thus ensure that this does not happen. We have found at least one clear example and a second probable case where the patient received an adequate graft on the basis of numbers (3.2 × 10⁸ nucleated cells/kg), but very poor growth was obtained in culture, and haematopoietic reconstitution did not occur. In vitro studies are thus a simple precaution to recognize those marrow donations where a paucity of stem cells may be expected to produce suboptimal results.

The study of pathologically disordered cell regulation is exemplified by the haematological malignancies. In the patients with preleukaemia there is a variable but usually marked reduction in plating efficiency accompanied by a disturbance in colony: cluster formation. The cytological detail of the individual cells in the aggregates and their ultrastructural examination remains an area for further development. Despite the apparent diagnostic value of culture studies in the preleukaemic patient, their place remains controversial, since clinical progression of the disease is still considered the essential criterion for initiating therapy. Nevertheless, if it can be shown that the combination of culture and morphology, perhaps combined with cytogenetics, will reliably predict the development of leukaemia, then the approach to treatment may be expected to change.

Similarly, bone marrow obtained from patients with acute leukaemia, both untreated and during relapse, grows with a characteristic pattern in agar. There is a marked reduction in overall growth or plating efficiency, colony formation is depressed and a variable number of clusters is evident. These findings are more prominent in lymphoblastic than in non-lymphoblastic leukaemia. It is notable that morphological relapse may only become evident many months after these abnormalities are manifest in culture, and the latter frequently appear at a time when the patients are clinically and haematologically normal. In this context we have encountered a leukaemic patient who, in complete remission, demonstrated a transient period of generalized acceleration in growth, evident many months before even colony: cluster ratio was disturbed, and without any pathological blasts being demonstrated in the blood or marrow; overt disease became obvious 6 months later. The explanation for this finding is uncertain; it might theoretically represent rapid expansion of the leukaemic clone or, alternatively, the response of normal progenitor cells to markedly enhanced humoral drive. The latter finding is favoured since the colony: cluster ratio was maintained, which contrasts with the more usual situation in leukaemia where the cells grow poorly or not at all, and tend to form clusters rather than colonies.

The best method of culturing leukaemic cells remains controversial, and despite reports that this is possible, there is no uniformity of agreement that the tumour cells will consistently grow in culture. Clearly, such an achievement would potentially provide a most sensitive measurement for the detection of residual leukaemia and be vital in defining early relapse. The scope of the method can be expanded by using methods to display the distinctive morphological features and the kinetic behaviour of leukaemic cells as compared with normal CFU. Detailed examination of cellular content within the clusters and the colonies as they exist in situ has been difficult, largely because no simple method has been available for this study, although recent experience with a different technique may help to resolve the problem. Similarly, the ability to store and maintain the viability of leukaemic bone marrow progenitor cells offers a practical opportunity to examine the influence of these cells in a number of different ways. For example, it should be possible to quantitate and characterize their production of CSF and to define its influence on normal CFU in culture. Conversely the response of these malignant cells to exogenous stimulating factors remains of interest. In addition, more sophisticated control mechanisms can be tested, such as interaction of the cellular level between normal and leukaemic cells using co-culture systems.

A third group of haematological neoplasms explored with this technique are those of the myeloproliferative syndrome. The pattern by which the marrow growth
changes as the disease evolves is now well defined and in chronic granulocytic leukaemia and myelofibrosis it is characterized by dislocation of the progenitor cell population from the marrow to the peripheral blood, a time sequence that has distinct diagnostic possibilities. Furthermore, a defect in plating efficiency and a change towards a leukaemic type of colony: cluster ratio develop as acceleration or blastic transformation occurs. Of equal interest is the potential of this technique to display the interrelationship between haematopoietic and stromal cells in vitro, thus providing a model with which to characterize myelofibrosis and perhaps to understand better the controversy about its pathogenesis. This argument has recently been given sharper focus by reports that stromal changes are secondary to the development of the haematopoietic lesion and are not part of the primary neoplasm.

The application of in vitro marrow culture to demonstrate humoral inhibitors is illustrated by examining marrow from patients with myeloma plated in either autologous or AB serum. Failure to depress colony formation excluded a humoral inhibitor as a major cause of the pancytopenia that may be present in these patients at diagnosis. The concept that defective haematopoiesis may be on the basis of cell: cell interaction can be tested by co-culture experiments. The latter possibility has its basis in observations that sub-sets of T lymphocytes may possess suppressor activities which will be reflected in pancytopenia or marrow hypoplasia.

Cloning techniques are helpful in formulating a functional or kinetic classification of the neutropenia. Thus it is possible to separate defective production clearly from situations where accelerated loss of mature cells occurs peripherally, as in classic immune neutropenia. In the latter situation, culture of the bone marrow shows normal or enhanced plating efficiency with retention of a normal colony: cluster ratio. This information is of relevance to the clinician who must direct investigation and therapy quite differently in the two groups of patients.

The application of in vitro culture systems to the study of patients with aplastic anaemia may help to unravel aetiological mechanisms in this heterogeneous group of diseases. In patients with a stem cell defect, poor plating efficiency is typical and is usually accompanied by a disturbance of colony: cluster ratio; here bone marrow transplantation remains the preferred therapy. In theory, any purely humoral cytotoxic factors could be identified by comparing cultures of progenitor cells in AB serum with those in autologous serum after initial washing to free the CFU of attached antibody. In the light of recent studies showing that immunologically mediated suppression of haematopoiesis may involve a subpopulation of patients with bone marrow aplasia requiring only immunosuppressive therapy, the ability to select these correctly by in vitro culture may come to assume still greater diagnostic importance. Clearly, then, the current objectives are to separate immunological lesions from those due to defective stem cells and to clarify the role of the micro-environment in the pathogenesis.

It is concluded that in vitro culture systems for the study of bone marrow have substantial clinical potential but require further development. Technically, there is the need to standardize methodology and refine morphological and ultrastructural studies of the cells and combine these with more sophisticated techniques such as cytogenetics. Physiologically, these methods provide a practical approach to the isolation and study of cellular and humoral effects in haematopoiesis, including interactions between stromal and haematopoietic stem cells. In clinical practice they have a role to play in the characterization of bone marrow grafts and form part of the current diagnostic tests for a range of haematological malignancies, including pre-leukaemia, early leukaemic relapse or the presence of minimal residual disease. Similarly in patients thought to have the myeloproliferative syndrome, the demonstration of CFU in the peripheral blood is a valuable ancillary to the diagnosis and provides one means of monitoring possible leukaemic transformation. The further applications of this method are numerous and include characterization of patients with neutropenia and bone marrow aplasia.

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REFERENCES