Medical Laboratory Haematology

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Preface

Many years involvement in teaching, in examining and in the practice of laboratory haematology, together with frequent requests to recommend suitable books, have indicated a need for a single volume that combines physiology, pathophysiology and the practical aspects. The aim has been to produce a textbook that is more than adequate for first qualifications and a sound basis for further study, which will also serve as a bench-book in the haematology department of a general hospital. We have therefore attempted to maintain a balance between the haematological disorders and the methods used in investigation, but in certain areas where technology is advancing rapidly, e.g. instrumentation or immunoassays using radioisotope or enzyme labels, we have confined the text to general principles and avoided recommending particular instruments and methods (or test kits!). This book is primarily for technologists, scientists and medical colleagues involved in laboratory haematology; and although inevitably the content is related to laboratories and examinations in the United Kingdom, we are confident that much of the content is relevant to laboratory haematology in other countries.

The photomicrographs have been restricted to those capable of illustrating morphological appearances in monochrome; those features that can be appreciated only by colour reproduction have been deliberately, though regrettably, omitted to maintain the book at a reasonable price. Clinical aspects and therapeutic considerations are included only when the laboratory is involved in monitoring treatment or when symptoms or physical examination indicate a particular line of laboratory investigation.

We are grateful to the many colleagues who knowingly or unknowingly have contributed in any way to the preparation of this book. Particular acknowledgement is made to the assistance of David Howard and the Department of Medical Illustration, St James's University Hospital for preparing the photomicrographs and to Alan Tunstill and the Department of Medical Illustration, Royal Hallamshire Hospital for the figures illustrating aspects of haemostasis. *Figure 13.1* is based on a scheme devised by Mr C.S. Scott. Mrs Wendy Mitchell and Mrs Eileen Murat skilfully transformed the draft into presentable form. To Butterworths for their patience, tolerance and encouragement, we extend our thanks.

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Robert G. Malia

Contents

Haemopoiesis

The blood volume of the normal adult accounts for approximately 7% of body weight or about 70 ml.kg^{-1}, of which 30 ml (45%) is occupied by the cellular elements and 40 ml (55%) is plasma. The cells are heterogeneous. They consist of erythrocytes, which are disc shaped, non-nucleated, non-motile and contain haemoglobin; the nucleated, motile leucocytes and the platelets. The leucocytes are further divided into granulocytes (neutrophils, eosinophils and basophils), lymphocytes and monocytes. Plasma contains many organic and inorganic compounds, notably proteins, electrolytes, substances absorbed from the gastrointestinal tract, hormones and products of metabolism.

All blood cells have a finite life span which may be shortened in disease. Plasma components, particularly proteins, have a determined rate of turnover which is often expressed as T½—the time during which half the substance is removed from the circulation and, in health, renewed. The 'turnover times' may be altered by disease.

The loss of cells through the normal processes of ageing, physiological consumption and, in certain circumstances, by loss from the body, is normally compensated by the process of renewal by the proliferation, maturation and release of new cells from the haemopoietic system—a process referred to as *haemopoiesis*. The haemopoietic system in the adult consists of the bone marrow and the lymphatic system. Other sites of haemopoiesis are active in the embryo and fetus.

Blood formation in the embryo and fetus

Haemopoiesis in the embryo and fetus takes place in three overlapping phases—the extraembryonic (in the yolk sac), the hepatic (and to some extent the spleen) and the bone-marrow phase. The yolk sac is the principal site in the early embryo and declines rapidly to be entirely replaced by the liver at about 2½ months' gestation. The hepatic phase is maximal between 4 and 5 months' gestation and then gradually declines towards term though some activity is still present at birth. The contribution of the spleen is maximal at the same time as the liver but starts at about 2½ months with a contribution that lasts until about 6½ months. The hepatic and splenic contribution is gradually taken over by that of the bone marrow which begins at 4 months and replaces the liver at term.

Erythropoiesis

Recognizable erythropoietic cells are found in the mesoderm of the yolk sac as early as the 14th day of development and produce erythrocytes for about 10 weeks. Initially morphology is similar to the megaloblasts associated with vitamin B_{12} and folate deficiencies. Normoblastic appearances are seen at 6 weeks. Mesodermal erythropoiesis is transferred to the liver which becomes the principal source of red cells during the second trimester of development. Hepatic erythropoiesis is macronormoblastic resulting in the production of macrocytic red cells. At 12 weeks' gestation circulating normoblasts are in the order of $50 \times 10^9 . \ell^{-1}$ with the red cells having an MCV of 180 fl. By 20 weeks the normoblast numbers have fallen to $1.0 \times 10^9 . \ell^{-1}$ and the MCV at 28 weeks is about 120 fl falling to a range of 100–120 fl at term. Maximum response to erythropoietin is demonstrable between 14 and 18 weeks.

The bone marrow contains recognizable erythroblasts at about 10 weeks, foci of development at 4 months, and by the 6th month has become the major site of erythropoiesis.

The transfer of erythropoiesis to different sites is associated with changes in the polypeptide chains of the globin molecule of haemoglobin. The production of α chains commences at about the 5th week and continues into, and throughout, adult life but is preceded by the formation of ζ chains. The precursors of the β and δ chains of adult haemoglobin are designated, in order of appearance, ε and γ chains. The normal haemoglobins found during erythropoiesis in the embryo and fetus are therefore:

$\zeta_2\varepsilon_2$ Gower I $\zeta_2\gamma_2$ Portland I $\alpha_2\varepsilon_2$ Gower II	}	Extraembryonic source detectable up to 12 weeks?
$\alpha_2\gamma_2$ Hb F (fetal Hb)	—	hepatic phase
$\alpha_2\beta_2$ Hb A $\alpha_2\delta_2$ Hb A_2	}	Bone-marrow phase

Hb F is detectable very early in embryonic life and at the 34th week constitutes about 90% of the circulating haemoglobin falling to between 50 and 85% at term. Hb F has a higher oxygen affinity than Hb A which would facilitate transport of oxygen across the placental membrane. Hb A is detectable in small amounts as early as 8 weeks of gestation but remains the minor haemoglobin component during fetal life. Hb A_2 appears at about 7 months' development as a minor Hb component.

Granulopoiesis

Though granulocyte formation can be observed in various embryonic and fetal tissues from about 7 weeks, the developing bone marrow is the major source of granulocytes starting at about 10 weeks. Relatively little activity is found until after 4–5 months' gestation. At 20 weeks circulating granulocytes number fewer than $1 \times 10^9 . \ell^{-1}$ but thereafter rise rapidly towards term.

Thrombopoiesis

A few megakaryocytes are found in the yolk sac at 6–7 weeks. They then develop in the liver and spleen and eventually the bone marrow at 13 weeks. Little is known of the production and numbers of platelets in the fetal circulation though at term the number is similar to that of the adult.

Lymphopoiesis

Lymphocytes have not been identified in the yolk sac. Formation commences in lymph plexuses at about 8 weeks and then in the thymus, lymph glands, spleen and in the bone marrow. About 2% of marrow cells are lymphocytes at 26 weeks rising to 8% at 30 weeks. Lymphocytes are found in the circulation as early as the 8th week reaching a maximum of $4–10 \times 10^9 . \ell^{-1}$ at 6 months and falling to $3 \times 10^9 . \ell^{-1}$ at term.

Bone-marrow structure and function

At term all the bone marrow apart from perhaps the terminal phalanges of the fingers and toes is active or 'red', and has a volume of about 1.4% of fetal weight.

The totally haemopoietic marrow of the new-born is gradually replaced with fat cells until adult development is attained when about half the marrow is fatty. The bone marrow is one of the largest organs of the body, having a volume of 30–50 ml. (kg body weight)$^{-1}$. Thus in a 70 kg adult the active marrow has a volume of about 1000–1750 ml. The gradual replacement of haemopoietic marrow by fat commences at the periphery of the skeleton and proceeds in the limb bones until, in the adult, active marrow is confined to the central skeleton.

The blood supply to the bone marrow is provided from a central artery which leads to branching arterioles and capillaries which open into sinusoids. The walls of the sinusoids consist of a single layer of cells without a basement membrane. The lining cells simply touch or overlap each other. This loose arrangement of sinusoidal endothelium separates the haemopoietic and fat cells of the extravascular tissue from the intravascular system and forms the marrow–blood barrier (MBB)[1]. The sinusoids are drained by a system of venules leading to the central vein.

The MBB controls the migration of formed blood cells from the extravascular haemopoietic compartment into the circulation and conversely probably influences the passage of essential nutrients, humoral stimulators and inhibitors of haemopoiesis, and possibly the selective trapping of circulating cells. In normal marrow the release of cells from the extravascular space is determined by the deformability of the cells. Thus segmented neutrophils can be aligned to squeeze between the loose endothelium. The release of erythrocytes is associated with removal of the nucleus (the so-called 'pitting' function)[2]. In certain abnormal states such as haemopoietic hyperplasia or when the architecture is altered by the presence of non-marrow elements the MBB is unable to control cell release effectively and immature cells can reach the circulation.

A similar situation arises when haemopoiesis occurs in tissues other than the bone marrow (extramedullary haemopoiesis) where an effective barrier does not exist. The effectiveness of the MBB is shown by its ability to control the release of about 6×10^{11} mature cells daily into the circulation while retaining the immature cells and maintaining the proliferative capacity of the marrow. The ability of the bone marrow to provide prompt delivery of cells to the circulation suggests that the MBB responds to humoral signals. Not only is there a prompt response but it is also selective in that erythrocytes are delivered when needed or granulocytes are released if required though some non-specific release of other cell lines may occur.

It has been suggested that the PCV is an important determinant of red-cell release into the sinusoids independent of erythropoietin levels[3,4]. Platelet release is an exception in that there is little marrow reserve, as indicated by the delay in recovery of circulating platelet numbers in individuals made thrombocytopenic. The megakary-

ocytes are located close to the sinusoidal endothelium and extend projections into the lumen and thus the circulation. A proportion of megakaryocytes enter the sinusoids intact and migrate to the lungs, where platelet formation takes place[5–7].

The extravascular space of the marrow is always filled and consists of haemopoietic cells, fat cells and non-haemopoietic cells. The distribution of collagen and reticulin is related to that of the blood vessels and sinusoids. Yellow marrow is predominantly fat and even red active marrow contains fat, particularly towards the centre of the marrow cavity. The degree of haemopoietic activity varies considerably, not only between different bones but between adjacent sites within the same bone. The lymphocytes of the adult bone marrow are distributed in nodules either as lymphoid follicles or lymphoid infiltrates. Follicles account for about 80% of the nodules with a reticulin framework, some having germinal centres[8].

In addition to its haemopoietic function the bone marrow, as part of the reticuloendothelial system, contains many macrophages which ingest particulate material, micro-organisms, extruded nuclei and in certain circumstances intact erythrocytes. The role of the marrow in removing effete red cells in normal subjects is not clear.

Cell proliferation

Proliferating cell systems vary in their growth characteristics in that the total number of cells may increase, remain constant or diminish. Haemopoiesis during intrauterine life is an example of an increasing cell population while haemopoiesis in the adult is normally in a steady state: production equals loss or destruction. Within a steady-state population, different cell types can vary greatly in the frequency of cell division; the relatively rapid turnover of erythrocytes requires that the earlier erythroblasts are in the cell cycle leading to mitosis, while others—e.g. the lymphocytes—divide only rarely and are not in cell cycle.

The cell cycle (*Figure 1.1*) is the period between the formation of a cell and the completion of mitosis in the newly formed cells and is known as the interphase. The development of techniques of microspectrophotometry for quantitation of DNA and autoradiography using radioactive DNA precursors has enabled the cell cycle to be divided in relation to the period of interphase when DNA synthesis occurs, the 'S' phase. Cells in the S phase are recognized by their uptake of radioactive thymidine as shown by autoradiography. The S phase is preceded by a postmitotic, pre-synthesis phase known as G_1 (or gap 1) in which the cells have a diploid (2n) DNA content. The post-synthesis, premitotic phase in which the cells have a 'tetraploid' (4n) DNA content is known as G_2 (or gap 2). Cells in S phase have a DNA content between 2n and 4n. Cells with a 2n DNA content that do not enter S phase are thought to have an extremely prolonged G_1 or are in a 'resting phase' designated G_0. However, G_0 cells may be stimulated to enter the cycle. The combined techniques of DNA quantitation and autoradiography therefore allows determination of the proportion of each cell type in G_1 (and G_0), S and G_2 phases. Further, the duration of G_1, S and G_2 phases can be measured and the 'labelling-index' of mitoses calculated. For further information, see Wickramasinghe[8].

Rates of cell proliferation may be adjusted by several mechanisms. Cells in G_0 may be stimulated to commence DNA synthesis, the period of G_1 may be shortened or prolonged as may S and G_2, and the duration of mitosis may vary. In certain disorders of haemopoiesis cell development may be arrested in G_2 with resulting cell death. Therapeutic drug regimens designed to reduce a cell population are often based on the stages of the cell cycle.

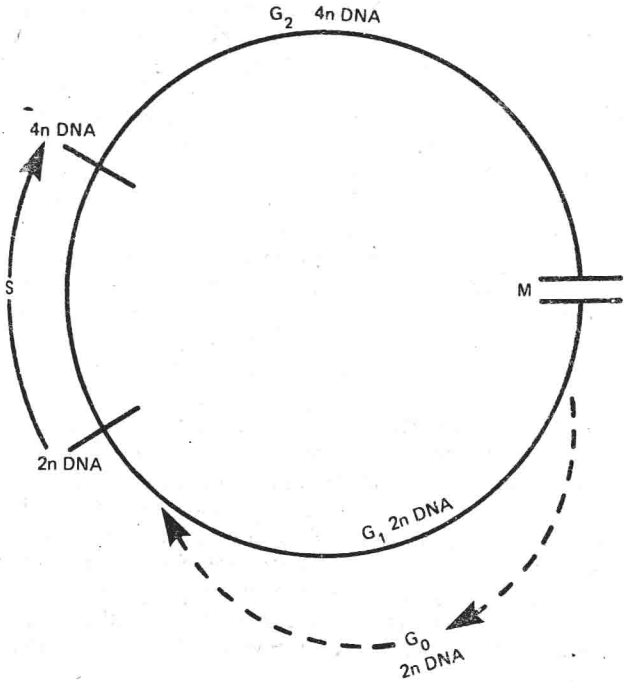

Figure 1.1 The cell cycle. The stages of cells in interphase are shown by: G_0 = cells not engaged in proliferation; G_1 = 'gap 1', the presynthesis phase of cells with a 2n DNA content; S = phase of DNA synthesis; G_2 = the post-synthesis/premitotic phase, the cells having replicated their DNA content though still having a diploid number of chromosomes; M = mitosis.

Regulation of haemopoiesis

General considerations

Though relatively small and transient changes in the number of circulating blood cells occur in health due to physiological responses related to physical activity, stress, hormonal influences or simply by eating, the number of cells is remarkably constant. This indicates that there must be a fine balance between cell loss and cell production. The control of cell production must involve the relatively crude differentiation at the stem-cell level in determining which cell line requires replenishment together with a 'fine adjustment' during mitotic replication, maturation and release into the circulation. Much of the early investigation was directed to the hormonal control of erythropoiesis by the characterization of erythropoietin (Epo) and was followed by the demonstration of hormonal control of other cell lines. For many years there has been controversy as to whether the blood cells develop from a single type of cell capable of differentiating into the various cell lines (a pluripotent 'stem cell') or whether each cell line is dependent upon a primitive precursor cell capable only of developing a single cell line. In recent years the development of techniques of bone-marrow culture and knowledge of the mechanisms regulating proliferation, differentiation and maturation have resolved some of these arguments. Nevertheless, by conventional methods of study the earliest identifiable precursors of blood cells are already differentiated or

'committed' blast cells with morphological, cytochemical, biochemical or immunological characteristics that permit their identification as myeloblasts, lymphoblasts, monoblasts, erythroblasts or megakaryoblasts. In 1961[9] the technique of inducing haemopoietic cell colonies in the spleen of lethally irradiated mice by the injection of bone-marrow cells provided a method for quantitative and functional studies of haemopoietic stem cells—the so-called 'colony-forming units—spleen' or CFUs—though the results had to be extrapolated to humans as at that time no equivalent technique was available for human stem-cell studies. The spleen colonies may be erythroid, granulocytic, megakaryocytic or of mixed cell lines.

The introduction of the technique of culturing the precursor cells of granulocytes and macrophages to form colonies (colony forming units in culture (CFU) or GM-CFU) in semi-solid medium containing a 'colony stimulating factor' (CSF) derived from other tissues, has allowed study of the factors controlling proliferation of the granulocytes[10]. More recently the growth of erythroid colonies in the presence of erythropoietin has provided a greater understanding of the control of erythropoiesis[11,12]. Megakaryocytes have also been studied by colony culture which suggests the existence of stem cells committed to megakaryocyte formation. Lymphocytes are also derived from the pluripotent stem cell with differentiation into the B and T classes after several cell divisions and, in the case of mature T lymphocytes, by possible modification in the thymus. Pluripotent progenitor cells can be cultured to produce colonies containing more than one lineage of haemopoietic differentiation—the CFU-GEMM (colony forming unit—granulocyte, erythrocyte, macrophage, megakaryocyte)[13]. These cells are earlier than the committed precursors such as BFU-E and GM-CFU (*see* below). The relationship between CFU-GEMM and pluripotent stem cells is not yet understood.

The CFUs cannot be identified by morphological criteria and studies are confined to the mouse. It was assumed and subsequently confirmed that a proportion of CFUs cells must be replicating to allow for the demand for differentiation to renew cell lines and at the same time retain by self-renewal the pluripotent stem-cell pool. It has been suggested that the 'immortal' stem cell is a 'fixed' and not easily transplanted cell whilst the CFUs cells are a transient population produced from the stem cell[14].

The pluripotent stem cell and its immediate descendant the CFUs is therefore seen as a self-renewing reserve compartment capable of feeding a proliferating compartment controlled by humoral influences. The proliferating compartment is an amplification stage possibly characterized by at least four cell divisions which is also associated with cell maturation and possibly differentiation, to provide a pool of non-proliferating cells in which further maturation continues before release into the circulation. Only a proportion of granulocytes are in the circulating pool; the remainder are sequestered in small vessels and constitute the marginal pool, from which they may enter the circulation under the influence of various physiological or pathological stimuli.

Erythropoiesis

Figure 1.2 shows the concept of orderly development of the erythroid series from stem cell to mature red cell and the role played by erythropoietin—the only specific regulator so far identified.

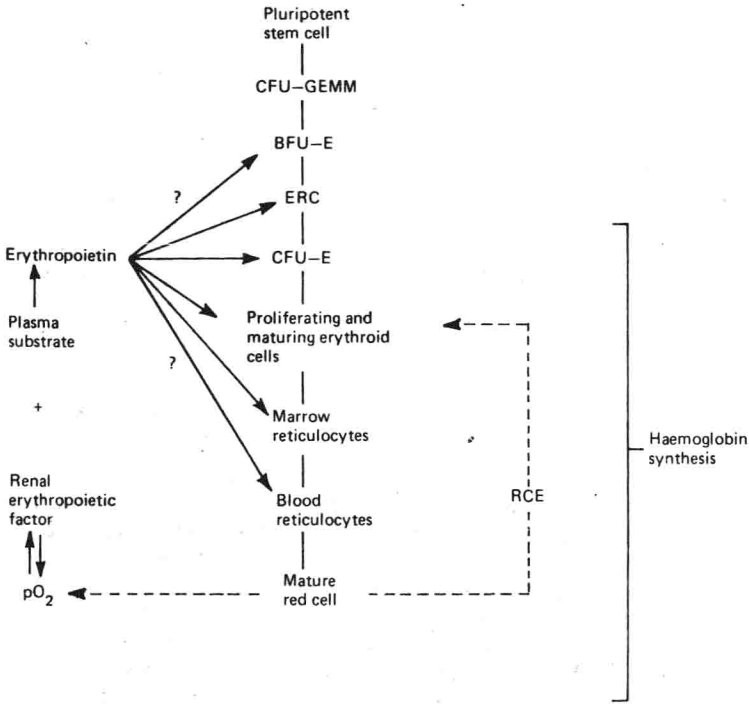

Figure 1.2 Schematic representation of erythropoiesis. CFU-GEMM = Colony forming unit—granulocyte, erythrocyte, macrophage, megakaryocyte; BFU-E = burst-forming unit—erythroid; ERC = erythropoietin-responsive cells; CFU-E = colony forming unit—erythroid; RCE = red-cell extract. ——— = stimulatory pathways; - - - - = inhibitory pathways.

The burst-forming unit—erythroid (BFU–E)

This cell is so called because of its ability to promote a number of erythroid clusters or 'bursts' after 5–6 days' culture and is the earliest cell committed to erythroid development. The number of BFU-Es is not influenced by erythropoietin though the number of 'bursts' shows a dose-related response to erythropoietin.

Erythropoietin-responsive cells (ERCs)

These are later stages of committed cells than BFU-Es and their number and differentiation is controlled by erythropoietin to give rise to small colonies of erythroid cells which have been shown to be derived from single cells.

The colony-forming units—erythroid (CFU–E)

When cultured from human marrow these reach size in 10–20 days with maturation and the synthesis of haemoglobin. CFU-E are probably a few cell divisions earlier than the first recognizable erythroid precursor—the pronormoblast.

Subsequent development is through the basophilic, early polychromatic and late polychromatic stages to the non-nucleated reticulocyte and finally to the mature

erythrocyte. Of these recognizable cells only the pronormoblast and the basophilic and early polychromatic cells are in cell cycle and normally four cell divisions transform one pronormoblast into 16 erythrocytes with a small loss of proliferating cells (so-called ineffective erythropoiesis). Maturation occurs both in the proliferating and in the non-proliferating pools.

There is evidence that DNA synthesis, and consequently cell division, is inhibited at a critical intracellular haemoglobin concentration.

Normally the nucleus of the normoblast is lost by extrusion rather than fragmentation (karyorrhexis), and the cell enters the circulation through gaps in the walls of the marrow sinusoids (diapedesis). The newly formed reticulocytes are apparently motile. ^{59}Fe studies have indicated that the time of development from pronormoblast to reticulocyte in the circulation is about 7 days, of which 3 days is the transit time in the marrow reticulocyte pool.

Erythropoietin

The suggestion that the rate of erythropoiesis is regulated by a humoral factor rather than a direct action of hypoxia was made over 70 years ago and was confirmed by inducing reticulocyte responses in normal animals by the injection of plasma from anaemic animals. The plasma factor was termed erythropoietin (Epo). Erythropoietin is a heat-stable, sialic-acid-containing glycoprotein present in very small amounts in normal plasma but excreted in higher concentration in urine. The molecular weight of human urinary Epo is about 60 000 daltons.

The production of erythropoietin in the normal human is controlled by the release of 'renal erythropoietic factor' (REF, erythrogenin) from the kidneys under the influence of hypoxia. REF generates erythropoietin by its action on a plasma substrate which is possibly synthesized in the liver. Thus the presence of anaemia produces tissue hypoxia in the kidney which results in increased REF production and consequently erythropoietin formation. Conversely, in hypertransfused subjects or when oxygen affinity of haemoglobin is reduced, erythropoietin is reduced and erythropoiesis suppressed. The biology of erythropoietin is reviewed by Graber and Krantz[15]. The effects of erythropoietin on red-cell kinetics may be summarized as follows:

1. Stimulation of erythropoietin-sensitive cells to differentiate into pronormoblasts.
2. Shortening of cell-cycle times or increase of the proportion of dividing cells among the unrecognized erythroid precursors.
3. Shortening the cell-cycle times of proliferating erythroblasts.
4. Shortening the total maturation time in the recognizable red-cell precursor pool.
5. Causing the production of macrocytes.
6. Promoting reticulocyte release from the marrow.

Erythropoietin also influences RNA and DNA synthesis, the transfer of iron from transferrin to erythroblast, the rate of haem synthesis and the rate of glycolysis via the pentose phosphate pathway. In other balanced biological processes there are usually inhibitory mechanisms, and inhibitors to erythropoietin have been isolated from tissue extracts and urine. Their precise role is not clear. Of particular relevance may be negative feedback influences of extracts from mature red cells and normal bone marrow. Of particular importance is the observation that extracts of mature red cells (RCE) inhibit the incorporation of tritiated thymidine by marrow cells *in vitro*. RCE may affect the recognizable proliferating cells by prolonging cell-cycle times and thus acts as a fine control on cell production.

Influence of other hormones on erythropoiesis

There is good evidence that hormones other than erythropoietin influence erythropoiesis in that endocrine deficiency is often associated with an anaemia that responds to replacement therapy. Furthermore the administration of the male hormone testosterone can induce erythrocytosis and conversely oestrogen administration depresses erythropoiesis. The mechanisms by which these hormones influence erythropoiesis is not entirely clear but there is evidence that much of their effect is achieved by influencing erythropoietin production by modifying either REF production or the plasma substrate, or even by influencing the effect of erythropoietin on the ERC compartment. *In-vivo* studies in mice have shown the influence of testosterone in inducing CFUs to enter the cell cycle.

Granulopoiesis

The earliest cell committed to granulocyte production is the granulocyte-macrophage colony forming unit, or GM-CFU, sometimes referred to as CFU-C (colony forming unit—culture). The growth of GM-CFU is stimulated by the presence of humoral factors known as colony stimulating factors or CSF which are produced by other cells in tissue culture. CSF may have different specificities in stimulating the various types of granulocyte. In practice GM-CFU refers to neutrophil lineage and the specific CSF is known as GM-CSF. Other forms of CSF are identified appropriately, e.g. eosinophil stimulating factor or EO-CSF.

Unlike erythropoiesis where radio-iron incorporation into haem can be used as a specific marker of cell maturation, the study of granulocytopoiesis lacks a satisfactory marker.

A major source of CSF is the phagocytic macrophage-cell population which itself is stimulated by CSF. This suggests that there must be a negative feedback system to prevent uncontrolled neutrophil proliferation. It had been suggested that this may be brought about by the existence of tissue-specific inhibitors known as chalones which can be isolated from granulocytes. The evidence for the inhibitory action of chalones is based on reducing the incorporation of tritiated thymidine into bone-marrow cells[16]. This concept has not gained total acceptance[17]. More recently the role of prostaglandins and lactoferrin as inhibitors of granulocyte production has gained acceptance[18].

Differentiation and maturation of granulocytes

The recognition of the myeloblast as the precursor of granulocytes is based on subtle morphological appearances supplemented by cytochemical characteristics, and more recently by electron microscopy and immunological differences. The long-held view that neutrophils, eosinophils and basophils are derived from a common myeloblast is not justified and each may well have a distinct myeloblast precursor.

Neutrophil proliferation and maturation is a good model for the concept of proliferating and non-proliferating compartments and subsequent release into marginating (MGP) and circulating pools (CGP) (*Figures 1.3*).

Transfer of neutrophils from the MGP to the CGP without an increase in the total granulocyte pool (TBGP) can be caused by physiological influences such as exercise or release of adrenaline which cause increased blood flow through the microcirculation. In such circumstances the increase in circulating neutrophils is not associated