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# APLASTIC ANEMIA

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## Pathophysiology and treatment

Edited by

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# Pathophysiology of acquired aplastic anemia

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# Stem cell defect in aplastic anemia

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## Abbreviations

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AA	Aplastic anemia
ADA	Adenosine deaminase
BFU-E	Burst-forming unit, erythroid
BL-CFC	Blast-colony forming cells
CAFC	Cobblestone area forming cells
CFU-E	Colony-forming unit, erythroid
CFU-GM	colony-forming unit, granulocyte/macrophage
CSF	Colony-stimulating factor
5-FU	5-Fluorouracil
G-CSF	Granulocyte colony-stimulating factor
GM-CFC	Granulocyte/macrophage colony-forming cells
HPP-CFC	High proliferative potential-colony forming cells
IL-1	Interleukin-1
IFN- $\gamma$	Interferon- $\gamma$
LTBMC	Long-term bone-marrow culture
LTC-IC	Long-term culture-initiating cell
LTRC	Long-term repopulating cells
M-CSF	Macrophage colony-stimulating factor
MIP1 $\alpha$	Macrophage-inflammatory protein $\alpha$
Mix-CFC/CFU-Mix	Mixed lineage multipotential colony-forming cells
NOD/SCID	Nonobese diabetic, severe-combined-immunodeficient mice
SCF	Stem cell factor
TGF $\beta$	Transforming growth factor $\beta$
TNF- $\alpha$	Tumor necrosis factor $\alpha$

## Normal stem cells

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### Definition

The need to continuously replace mature cells in the blood requires the production of about  $10^{11}$  new cells daily in a normal adult, and even more in response to hemopoietic stress. It is known that all these cells are derived from a common ancestor population, the pluripotential stem cells (Lajtha, 1983). The usually accepted definition of stem cells is based on three characteristics: first, their marked capacity for proliferation, as just illustrated, and, second, their potential to undergo differentiation to produce all the lymphohemopoietic mature cell types (Metcalf, 1988). Third, and classically, stem cells are also defined by their reported capacity for self-renewal, i.e., the capacity to generate new stem cells, with the implication that they are able to regenerate their own population (Lajtha, 1983; Metcalf, 1988). As we will discuss later, it is mainly this latter concept that has to be discussed in the context of aplastic anemia (AA).

### Regulation

Stem cells comprise only a small minority, between 0.01 and 0.05%, of the total cells found in bone marrow. At least 95% of the hemopoietic cells fall into morphologically recognizable types. The remaining, with nonspecific morphological features, require phenotypic and functional characterization. They encompass not only the stem cells but also their immediate progeny, the progenitor cells which were first characterized by their ability to develop in vitro in response to the colony-stimulating factors (CSF) (Metcalf, 1988). As the hemopoietic tissue is a continuum of differentiating and proliferating cells, the boundaries between different primitive cell populations are ill-defined. However, it is generally agreed that commitment, i.e., a decision to enter a particular differentiation lineage (therefore restricting the multipotentiality of the stem cells), distinguishes between stem and progenitor cell populations. Such commitment appears to be irreversible. For example, macrophage progenitors which are genetically manipulated to present the erythropoietin receptors still develop into macrophages following stimulation with erythropoietin (McArthur et al., 1995). In the converse experiment, erythroid progenitors induced to express the receptor for macrophage colony-stimulating factor (M-CSF) develop into erythroid cells in response to this cytokine (McArthur et al., 1994). In contrast, little is known about the commitment process itself and, at present, the argument rages whether such an event is dictated internally by the cell-driven program, or is the response to external, i.e., environmental, stimuli (Jimenez et al., 1992; Ogawa, 1993). These concepts are important to the under-



standing of AA, since stem cells, which must supply mature functional cells for a lifetime, must be protected from the competing demands for mature cells in response to physiological or pathological needs. The rapid response of the hemopoietic tissue is met by the more mature, differentiation-restricted progenitors; for example, BFU-E cells (burst-forming unit; erythroid) in response to hypoxia or blood loss, or granulocyte/macrophage colony-forming cells (GM-CFC) in response to infection. These, and equivalent cell populations in the other lineages, are largely controlled by growth factors. However, the steps that *generate* these progenitors from stem cells are, at present, unknown. In the context of AA, it is interesting to speculate whether, in some cases, the 'protection' mechanisms that may act to protect the stem cell population from exhaustion are defective. As stem cells are lodged within bone marrow stroma, it is generally assumed that those stromal cells produce and may present a membrane-bound form, or release a large number of regulatory cytokines, including stimulatory molecules such as interleukin-1 (IL-1), M-CSF, GM-CSF, G-CSF, IL-6 and stem cell factor (SCF), as well as inhibitory cytokines such as transforming growth factor  $\beta$  (TGF $\beta$ ) and macrophage-inflammatory protein  $\alpha$  (MIP1 $\alpha$ , for review see Lord et al., 1997). However, whether they have a role in stem cell differentiation is not known. Some do have a role, at least in vitro, in their survival and proliferation (Fairbairn et al., 1993). It may very well be that regulatory factors crucial for the commitment to differentiation are still unknown; such factors may have more in common with those which regulate embryonic and fetal development than with the 20–30 cytokines known to regulate the proliferation, maturation and function of the committed hemopoietic progenitors and their developing progeny (Lord et al., 1997).

### The experimental study of primitive hemopoietic cells

While assays for colony-forming cells detect mainly progenitor cells, transplantation experiments define stem cells by their function, i.e., their capacity to repopulate permanently the hemopoietic tissue (Table 1.1). The long-term repopulating cells (LTRC) can, at present, be assayed only in experimental systems.

Currently, the most primitive human cell that can be assayed in vitro is the long-term culture-initiating cell (LTC-IC) (Table 1.1). This cell has certain stem cell characteristics, but it is not yet clear how it is related to the human stem cell. However, Ploemacher (1994) showed that murine LTC-IC (assessed as cobblestone area forming cells or CAFC) were able to repopulate irradiated mice and could, therefore, be regarded as equivalent to the mouse repopulating cell. A number of animal models have been developed for transplantation studies. Sublethally irradiated, severe-combined-immunodeficient, nonobese diabetic (NOD/SCID) mice were used to test the engraftment and repopulating potential

Table 1.1. Assays for primitive stem and progenitor cells

Cells	Assay	Incidence in bone marrow	References*
Long-term repopulating cells (LTRC)	Reconstitution of hemopoietic tissue	$1/(5 \times 10^4 - 10^5)$	Lord et al., 1997; Ploemacher, 1994
Long-term culture initiating cells (LTC-IC)	Generation of progenitor cells (CFC after 5–8 weeks of culture)	$1/(10^4 \text{ to } 2 \times 10^4)$	Lord et al., 1997; Ploemacher, 1994; Testa et al., 1996
Also called cobblestone area forming cells (CAFC)	Generation of a cobblestone area of cell proliferation		
Multipotential colony-forming cells (CFC)** HPP-CFC BL-CFC Mix-CFC	Colony formation in vitro	$1/(5 \times 10^4 - 10^5)$	Lord et al., 1997; Metcalf, 1988
Bipotential CFC	Colony formation in vitro	$1 - 2/10^3$ according to lineage	Lord et al., 1997; Metcalf, 1988

Notes:

\* Mostly reviews are quoted.

\*\* Definitions in the text and beginning of chapter.

of putative human stem-cell populations. However, limiting dilution repopulation assays indicate that the frequency of a NOD/SCID mouse repopulating cell is 1 in  $10^6$  cord blood mononuclear cells, whereas 1 in  $3 \times 10^3$  to  $10^4$  mononuclear cells was an LTC-IC (Pettengel et al., 1994). Clearly, the human repopulating cells, as assessed in the NOD/SCID model, appear to be more primitive than the human LTC-IC. Furthermore, a gene-transfer study using a retroviral adenosine deaminase (ADA) vector showed that 30–40% of colony-forming cells and LTC-IC could be transduced with the ADA vector, but, when the cells were transplanted into NOD/SCID mice, none of the colony-forming cells generated were positive for ADA. Although high numbers of colony-forming and mature cells were obtained, it seems that the transfected cells contributed little to the graft, and the cells responsible for repopulation were not transfected. This further indicates that the repopulating cell may be more primitive than the LTC-IC. On the other hand, potentially lethally irradiated mice can be rescued from hemopoietic death by  $5 \times 10^4$  to  $10^5$  bone marrow cells (Lord et al., 1997). It is not clear whether the larger numbers of human cells required to rescue the irradiated NOD/SCID mice mean that there are fewer stem cells in humans than in mice. However, there are problems with the maturation of human cells in those mice (Larochell et al., 1996), which suggests that the relatively low incidence of LTRCs in this system may be an assay-driven paradox. The clonogenic *in vitro* assays detect mainly the progenitor cells, which are more mature than stem cells. However, because of the continuous spectrum of proliferation and differentiation in the hemopoietic tissue, some of the clonogenic assays may partially overlap with the stem cell compartment. The blast colony assay (BL-CFC) and the high proliferative potential colony assay (HPP-CFC) are within this category.

### **Selection of primitive cells by phenotype**

It is possible to separate the most primitive cells from their close progeny of progenitor cells. The former have distinct cell membrane markers (Table 1.2) and are also characterized by low metabolic activity. This latter feature allows primitive cells to be isolated by negative selection, using dyes such as rhodamine-123, which concentrates in active mitochondria, or nucleic acid dyes like Hoechst 33342 (Ratajczak and Gerwitz, 1995; Spangrude, 1994).

One of the most useful membrane markers for the selection of primitive cells has been the CD34 antigen, and this feature has been exploited in a number of different positive cell-selection procedures (de Wnnter et al., 1995). However, the cells that are CD34+ comprise a wide population, encompassing stem cells, progenitor cells and more differentiated hemopoietic cells. In fact, only 0.1–1% of the CD34+ cells have the most primitive phenotype, while about 10–30% are progenitor cells, and the rest are more differentiated cells (Table 1.3).

**Table 1.2.** Phenotypic markers of primitive hemopoietic cells

Stem cells	Progenitor cells
CD34+	CD34+
CD38–	CD38+
CD33–	CD33+
Lineage–	Lineage+
HLA-DR– or weakly +	HLA-DR– or weakly +
CD71–	CD71+
Thy 1 <i>low</i>	Thy 1+
CD45RA <i>low</i>	CD45RA+
<i>c-kit</i> +	<i>c-kit low</i> or –

*Note:*

Reviewed in de Wynter et al., 1995; Lord et al., 1997; Spangrude, 1994; Testa et al., 1996.

**Table 1.3.** Percentage of colony-forming cells (CFC) in the different CD34+ subpopulations expressing stem and progenitor cell phenotype

Phenotype	Percentage of cells	Percentage of CFC
CD34+38+DR+	90	31
CD34+38+DR–	4	N.D.
CD34+38–DR+	6	1.0
CD34+38–DR–	0.3	0.2

*Note:*

Data calculated from Wynn et al., 1998; N.D. = not determined.

### How many stem cells get to express themselves?

Recently, a study demonstrated that one injected cell with a 'stem cell' phenotype is able to reconstitute long-term hemopoiesis in an irradiated mouse (Osawa et al., 1996). The proportion of mice injected with single cells that were reconstituted agrees with the expected proportion (about 20%) of cells seeding in the bone marrow (Testa et al., 1972). Other transplantation studies with marked murine cells have also demonstrated that monoclonal or oligoclonal hemopoiesis may be observed for long periods of time (Capel et al., 1988; Keller and Snodgrass, 1990). Only limited data are available in larger mammals; in experiments with cats, small numbers of syngeneic stem cells are able to maintain hemopoiesis (Abkowitz et al., 1995).

In humans, normal hemopoiesis is polyclonal, and polyclonal hemopoiesis is also usually observed following allogeneic transplantation. Nevertheless, there are anecdotal reports of oligo- or monoclonal hemopoiesis after allogeneic transplantation. This was observed in two out of 12 cases by examining X-chromosome-linked polymorphisms, one of them limited to myeloid cells and the other also comprising lymphoid cells (Turhan et al., 1989). Unfortunately, these observations were made days or weeks after transplant, and the long-term features of hemopoiesis in those patients are not known. However, oligoclonal hemopoiesis, as determined by cytogenetic marks on atomic bomb survivors, may be observed for several years (Amenomori et al., 1988). In one patient, a single identifiable clone provided about 10% of all the lymphohemopoietic cells for an observation period of 10 years, in the absence of any detectable sign of abnormal hemopoiesis (Kusunoki et al., 1995).

Recent studies of normal subjects showed that about 30% of females aged 70 years or older had oligoclonal hemopoiesis in the myeloid, but not the lymphoid lineages (Champion et al., 1997; Gale et al., 1997). It is not clear whether this is caused by altered regulation of cell production or a limited supply of stem cells in the aged. These data, taken together, suggest that only a few stem cells may, under normal steady state, be needed to maintain normal hemopoiesis. They also confirm the early concept that the stem cell population is normally quiescent, and that only a fraction of their vast reserve population needs to express itself, differentiating and giving rise to progeny. In the context of AA, these data also suggest that a mere reduction of stem cell numbers may not be sufficient to cause this syndrome. It is also important to consider how many of the available stem cells are likely to proliferate in AA.

Progressive telomere shortening of CD34+ occurs with age (Vaziri et al., 1994), and we have shown, in paired studies of donors and recipients of allogeneic transplantation, that the telomere length of the recipient's blood cells is significantly shorter than that of their donors. Such shortening is equivalent to that observed during 15 years of normal aging and, in the worst cases, is equivalent to 40 years (Wynn et al., 1998).

Although we do not yet know the molecular mechanisms of this phenomenon, we attribute the accelerated telomere shortening to proliferation stress. If stem cells age, do they conserve their capacity for self-reproduction after the hemopoietic system has reached its adult size? Cultures of human hemopoietic cells have achieved marked expansion of CFC and of LTC-IC (reviewed in Testa et al., 1999), but it is more problematic to assess whether stem cells have increased in number, as assessed by an increase in their capacity to regenerate hemopoiesis. While a primitive phenotype may be conserved, the repopulation capacity may be decreased (Albella et al., 1997). Because of this, it is not known whether the numbers of cells needed for transplantation will be the same when using freshly harvested cells or cells expanded in vitro. Experiments on mice

indicate that 6-fold to 50-fold more in-vitro-generated GM-CFC are required to achieve an equivalent number of leukocytes in the blood (Albella et al., 1997). Therefore, it is doubtful that significant expansion of the stem cell population has been achieved. This may not be surprising since the stimulatory cytokines used in those experiments are those known to act on the progenitor cell populations.

Extensive data have also been obtained from experimental systems and patients. Such data indicate that, following serious cytotoxic injury, the stem cell population recovers to a lesser extent than more mature populations, and remains at markedly subnormal levels for the rest of the experimental animal's life, and for several years at least in patients (reviewed in Testa et al., 1996). Progenitor and maturing cell populations have evolved in response to selective pressures that stimulate hemopoiesis, such as infection and blood loss. In contrast, the use of irradiation and the cytotoxic drugs that kill stem cells were developed recently, in the twentieth century; therefore, it is not surprising that they have not developed mechanisms to normalize their numbers after injury. Fortunately, as discussed above, first, their normal numbers far exceed those needed for a normal life span, and second, an adequate output of mature cells may be reached even with a severely restricted stem cell compartment. However, it is apparent that the concept that hemopoietic stem cells in the adult have the capacity to self-reproduce has to be revised. Perhaps it is more realistic to think that while stem cells are characterized by a very extensive proliferation capacity, each cell division results in some stem cell aging. Thus, while operationally the daughter cells may still be defined as stem cells, they are not identical to the parent cell.

## **Aplastic anemia stem cells**

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### **Functional assessment of AA hemopoietic progenitor cells**

Early work from the 1970s, with clonogenic cultures using unpurified bone marrow mononuclear cell preparations and various conditioned media as a source of colony-stimulating activity, demonstrated a reduction or absence of late and early colonies (CFU-GM, CFU-E, BFU-E and CFU-Mix) in patients with AA (Barrett et al., 1979; Hara et al., 1980; Kern et al., 1977). Although variation in colony numbers was seen between individual patients, there was a uniform lack of correlation with disease activity in terms of peripheral blood neutrophil count or marrow granulocytic precursors. Numbers of peripheral blood colonies were at least 10-fold less than bone marrow colonies and more often undetectable. More recent studies using purified (CD34+) hemopoietic cells and recombinant hemopoietic growth factors in clonogenic culture confirm the reduced numbers

of all marrow progenitor cells (Maciejewski et al., 1994; Marsh et al., 1991; Scopes et al., 1996).

The long-term bone-marrow culture (LTBMC) system has been used by several groups to (1) evaluate the earlier stages of hemopoiesis and (2) assess the ability to form a normal stromal layer, the *in vitro* representation of the marrow micro-environment. All studies of AA patients have demonstrated a marked defect in hemopoiesis, as manifest by a severe reduction in, or cessation of, the generation of hemopoietic progenitor cells within the system (Bacigalupo et al., 1992; Gibson and Gordon-Smith, 1990; Holmberg et al., 1994; Marsh, 1996; Marsh et al., 1990). A similar pattern is seen in untreated patients, whether with severe or nonsevere disease, and treated patients who have responded hematologically to immunosuppressive therapy (Marsh et al., 1990).

The formation of the stromal layer is normal in most patients with AA (Gibson and Gordon-Smith, 1990; Marsh et al., 1991), although one study reported a lack of stromal confluency in almost half the patients, and that this was associated with a longer duration of disease (Holmberg et al., 1994). Using a different short-term culture system, Nissen and colleagues (1995) reported impairment of stroma formation at 2 weeks but most became confluent at the standard long-term culture time. In contrast, some AA patients form a confluent layer more rapidly than normal (Marsh et al., 1990).

The defect in hemopoiesis seen in LTBMC may reflect either a failing in the stem cell compartment with a deficiency of primitive cells with marrow-repopulating ability, or a dysfunctional microenvironment. Cross-over LTBMC experiments allow separate examination of these two components. Using AA marrow adherent-cell-depleted mononuclear cells, one group demonstrated defective generation of CFU-GM when the cells were inoculated onto normal irradiated LTBMC stromal layers (Marsh et al., 1990). In contrast, normal stromal function in AA patients was demonstrated by normal numbers of CFU-GM generated from normal marrow mononuclear cells when inoculated onto irradiated stromal layers from AA patients, except in one patient in whom a defective stroma was demonstrated. A second group showed similar results, assessing BL-CFC generation on irradiated stromal layers in AA (Novotski and Jacobs, 1995). Furthermore, a similar pattern was seen using purified CD34+ cells as the inoculum, in that the stroma in AA patients supported generation of normal CFU-GM (Marsh et al., 1991) or BL-CFC (Novotski and Jacobs, 1995) from normal marrow CD34+ cells, and purified AA CD34+ cells failed to generate normal numbers of CFU-GM on normal stromas. Hotta and co-workers (1985) had previously demonstrated abnormal stromal function in three out of nine AA patients, although their stem cell function was not examined.

The results of these cross-over experiments indicate a deficiency or defect in primitive cells with marrow-repopulating ability, which in normals had previously been shown to exhibit the CD34+, CD33- phenotype (Andrews et al.,

1989) and within which population LTC-IC are found. Although not all patients form a confluent stroma, in those patients in whom stromal function has been evaluated, in terms of their ability to support the generation of hemopoietic progenitors, the majority function normally. A reported isolated deficiency of a growth factor or increased expression of an inhibitory cytokine (Holmberg et al., 1994) appears not to affect the physiological function of the stroma, as assessed by the long-term marrow-culture system.

### **Phenotypic quantitation of AA hemopoietic (CD34+) cells**

The percentage of bone marrow CD34+ cells is significantly reduced in AA patients compared with normal steady-state bone marrow, with median values of around 0.5%, but with a wide range seen from zero to values falling within the normal range (Maciejewski et al., 1994; Marsh et al., 1991; Scopes et al., 1994). Analysis of the CD34+ subpopulation reveals a significant reduction in the immature CD34+,33- cells, as well as the more mature CD34+,33+ cells (Scopes et al., 1994). A lack of correlation between these compartments and disease severity was reported by one group. Although a second group reported significantly higher percentages of CD34+ and CD33+ cells in patients with recovered AA, almost half the patients had persistently reduced values (Maciejewski et al., 1994). In other words, extreme variability of results was seen among patients who had recovered hematologically after immunosuppressive therapy. It should be remembered that the CD34+ compartment comprises a very heterogeneous collection of cell types in terms of their stage of differentiation, the majority of which comprise the more lineage-restricted progenitors, with the more primitive progenitors comprising only a very small proportion of the CD34+ cells. It appears that, in AA, the CD34+ population contains a much smaller proportion of very primitive cells, with a relative over-representation of more mature progenitors.

AA CD34+ hemopoietic cells have also been shown to be dysfunctional (Scopes et al., 1996). Although marrow mononuclear cells from AA patients consistently produce lower numbers of colonies compared with normal, when the reduced numbers of CD34+ cells in AA bone marrow are considered there is no significant difference in clonogenic potential. However, when purified AA CD34+ cells cease to be influenced by accessory cells, their clonogenic potential is significantly reduced, indicating defective function. From the same study, the effects of various hemopoietic growth factors in isolation or in combination on the clonogenic potential of AA marrow cells was investigated. It was shown that the addition of granulocyte colony-stimulating factor (G-CSF) in vitro was able to correct the dysfunction of AA CD34+ cells to normal in terms of their clonogenic potential. Thus, in AA there appears to be both a deficiency and a dysfunctionality of marrow CD34+ cells.

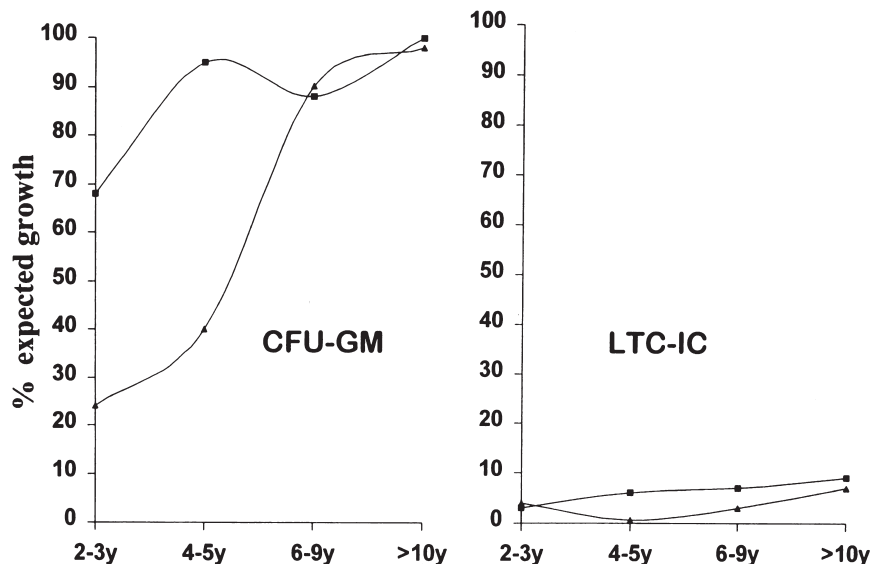


### Assessment of the long-term marrow-repopulating ability of AA hemopoietic cells

As discussed earlier, the LTC-IC and CAFC assays represent modifications to the LTBM system to permit quantitation of these primitive hemopoietic cells. Maciejewski and colleagues (1996) demonstrated, by limiting dilution analysis, reduced clonogenic potential of LTC-IC in two patients; however, for other AA patients examined, limiting dilution analysis was not possible because of low cell numbers. Instead, results of LTC-IC frequency were extrapolated from week-5 clonogenic cells from bulk cultures and the numbers divided by the average proliferative potential of single AA LTC-IC, based on the small number of formal limiting dilution assays. Using this methodology, the frequency of LTC-IC was reduced compared with normal controls (AA patients had 0.024 colonies/ $10^5$  mononuclear cells compared with 7.8 for normal controls). Furthermore, LTC-IC remained subnormal in those cases, despite achieving normal or near-normal blood counts. LTC-IC were also qualitatively abnormal, demonstrating a markedly reduced clonogenic potential. Schrezenmeier and colleagues (1996) have also measured the frequency of LTC-IC but used the CAFC as the endpoint for scoring LTC-IC at week 5 instead of the generation of colony-forming cells. They demonstrated a reduction in CAFC in AA patients (mean frequency of CAFC was 6.6/ $10^5$  mononuclear cells (mnc) compared with 84.4 for normal controls). The frequency of LTC-IC is notably higher than reported by Maciejewski et al. (1996), raising questions as to whether the two assay systems are exactly comparable, and whether the CAFC assay detects a somewhat more mature progenitor cell than the LTC-IC (Weaver et al., 1997). In summary, these studies indicate a deficiency in LTC-IC in AA patients, which would account for the deficient marrow-repopulating ability seen in LTBM.

Podesta and colleagues (1998) have compared the frequency of late hemopoietic progenitors and LTC-IC in AA patients after immunosuppressive therapy with that in AA patients who have undergone successful allogeneic bone marrow transplant (BMT), over a follow-up period of up to 20 years. Although all patients had achieved normal blood counts, bone marrow cellularity and numbers of CFU-GM, BFU-E and CFU-Mix remained subnormal, but there was an even more striking reduction in LTC-IC, equally in transplanted patients and those who had received immunosuppressive therapy (see Figure 1.1). The pattern of recovery of CFU-GM between the two groups was different, with a more rapid normalization of CFU-GM in transplanted patients over a period of 2 years. In contrast, patients treated with immunosuppressive therapy displayed a more prolonged pattern of recovery of CFU-GM over 5–6 years, which may reflect an ongoing process of suppression of hemopoiesis among these patients (see Figure 1.1). From these results, it appears that even a markedly reduced stem cell reservoir (as assessed by LTC-IC frequency) is able to maintain steady-state hemopoiesis, although this

Figure 1.1. Pattern of growth of mixed myeloid cultures (colony-forming unit granulocyte/macrophage or CFU-GM) and long-term culture-initiating cells (LTC-IC) over time following treatment with immunosuppressive therapy (▲) and bone marrow transplantation (■). Numbers are expressed as percentage of expected growth; 100% refers to a median normal of  $58/10^5$  mononuclear cells (mnc) for CFU-GM and  $34/10^6$  mnc for LTC-IC; y = years from treatment (reproduced from Podesta et al., 1998, with permission).



may not be maintained under conditions of hemopoietic stress. In terms of the quality of the LTC-IC, in the transplanted patients LTC-IC generated normal numbers of colony-forming cells at week 5. In contrast, the proliferative potential of LTC-IC was reduced in patients treated with immunosuppressive therapy, compared with normal controls. This would seem to indicate a qualitative abnormality in stem cells derived from patients who recover autologous hemopoiesis after immunosuppressive therapy compared with the normal quality of stem cells (LTC-IC) grown from AA patients receiving an allogeneic stem cell transplant. Persistence of this abnormality may be one explanation for the risk of relapse of AA or later clonal evolution. An alternative explanation for these results is that most stem cells (and LTC-IC) in AA are unable to enter the cell cycle and proliferate normally. This may be compensated for by increased replicative pressure on the more mature hemopoietic progenitor cells (see 'Analysis of telomeric DNA length in AA', p. 16). From a practical viewpoint, the altered cell cycling status of AA stem cells would impact on an attempt to quantitate LTC-IC and make direct comparison of LTC-IC frequency with that of normal controls difficult. Hence LTC-IC assays may not be suitable for the quantitation of very primitive hemopoietic cells in AA.

Very little is known about the kinetics of stem cell proliferation in AA. Maciejewski and colleagues (1994) examined the expression of *c-kit* on AA CD34+ cells, on the basis that in normal marrow CD34+ *c-kit*+ cells contain the highest proportion of cycling cells. Cell cycle analysis was not performed on AA CD34+ cells, but they showed that the percentage of *c-kit*+ cells among the CD34+ cell population was reduced, suggesting that in AA fewer CD34+ progenitors are

cycling. Preliminary work by Gibson and colleagues (1996) has demonstrated reduced regeneration of progenitors from 5-fluorouracil-treated (5-FU-treated) AA bone marrow cells inoculated onto irradiated LTBM stromal layers compared with normal 5-FU-treated cells, and that colonies were produced for only 2–4 weeks. This suggests defective or deficient numbers of primitive noncycling stem cells in AA, and also that the finding of reduced or absent LTC-IC in AA may also reflect abnormal proliferation and differentiation kinetics of the stem cells.

### **Mobilizing potential of AA progenitor cells**

It is well established that primitive hemopoietic progenitor cells (including true stem cells from long-term follow-up of allogeneic peripheral-blood stem-cell transplants) can be mobilized from the bone marrow of normal donors using G-CSF (To et al., 1997). However, it may be possible to mobilize residual stem cells from AA patients. Collection and cryopreservation of mobilized stem cells may allow the subsequent use of intensive immunosuppression followed by reinfusion of the stem cells. One group has attempted to collect mobilized blood progenitor cells in AA patients following treatment with antilymphocyte globulin and cyclosporin and 3 months of daily G-CSF (Bacigalupo et al., 1993). The median number of CD34+ cells collected was  $1.8 \times 10^6/\text{kg}$  (range 0.27–3.8) and median CFU-GM  $3.9 \times 10^4/\text{kg}$  (range 0–39). Colony growth was only obtained on leukaphereses performed between days +33 and +77. There was marked patient variability in terms of mobilizing ability, but in some cases sufficient CD34+ cells were obtained for potential autologous transplantation. It is not known, however, whether any LTC-IC can be isolated using this procedure, and, so far, we are not aware of any report using this approach to treat AA patients.

### **Apoptotic properties of AA CD34+ cells**

It has recently been demonstrated that AA CD34+ marrow cells are more apoptotic than normal CD34+ marrow cells. In addition, there appears to be a correlation between the percentage that is apoptotic and disease severity, and also between the percentage of CD34+ cells present (Philpott et al., 1995). Increased apoptosis may be an important contributory factor to the stem cell defect in AA. Maciejewski and colleagues (1995*a*) had shown that AA CD34+ cells show increased expression of Fas-antigen and that tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) upregulate the expression of Fas-antigen on normal CD34+ cells (Maciejewski et al., 1995*b*). Whether the Fas system is involved in apoptosis in AA remains to be determined. This topic is discussed in detail in Chapter 4. The ability of hemopoietic growth factors such as G-CSF to suppress apoptosis may be an important factor in the effect of G-CSF in vitro and in vivo in AA patients.

### Analysis of telomeric DNA length in AA

As discussed earlier, normal hemopoietic stem cells demonstrate progressive telomere shortening with age. A recent study of patients with AA has shown significantly shorter mean telomere length in both granulocytic and mononuclear cell fractions compared with age-matched controls, suggesting some loss at the level of the hemopoietic stem cell (Ball et al., 1998). The degree of telomere loss was proportional to disease duration, and amounted to a loss of 216 base pairs (bp) per year in addition to the normal age-related loss of 36 bp/year. In those patients who had achieved normal blood counts after treatment, the rate of telomere loss had stabilized. It may be that the remaining hemopoietic progenitor cells need to undergo a greater number of cell divisions in order to generate sufficient mature blood cells. This may reflect stem cell loss caused by an increase in apoptosis of stem cells and primitive progenitor cells, or direct immune destruction of these cells. An increase in the replicative capacity of hemopoietic progenitor cells may account for the increased telomere loss in AA patients.

### Conclusion

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It is now apparent that only a few stem cells are required to maintain normal steady-state hemopoiesis. Normal stem cells exhibit a progressive shortening of telomeric DNA with age, so their self-replicative capacity is not preserved with time. Furthermore, following injury to the stem cells from chemotherapy, for example, the stem cell reservoir does not recover to normal in contrast to the more mature progenitor cells. For these reasons, the classic concept of the hemopoietic stem cell with unlimited self-renewal capacity has been revised, so that with each cell division and after marrow injury, the daughter stem cell is not identical to the parent stem cell in terms of replicative capacity. This concept is important when attempting to define the nature of the hemopoietic defect in AA where there is failure of normal hemopoiesis. Both a deficiency and a dysfunction of hemopoietic progenitor (CD34+) cells occurs, and, with hematological recovery, numbers of mature progenitor cells can return to normal but a deficiency and a dysfunction remains at the level of the primitive progenitor cells (LTC-IC). AA patients also show an exaggeration of the normal pattern of telomere shortening, which may contribute to the markedly reduced replicative capacity of the stem cells. This may occur because of an increased loss of stem cells and committed progenitor cells by apoptosis, or by direct immune destruction. The pattern of recovery of hemopoietic progenitor cell numbers in patients with idiopathic AA is similar to that seen following injury to normal bone marrow after chemotherapy, but the exact mechanism behind the injury to the stem cells is poorly understood and likely to be very different.

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