Aplastic Anemia: Evidence for Dysfunctional Bone Marrow Progenitor Cells and the Corrective Effect of Granulocyte Colony-Stimulating Factor In Vitro

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We investigated the effects of granulocyte-macrophage colony-stimulating factor, interleukin-3, stem cell factor, interleukin-6, and granulocyte colony-stimulating factor (G-CSF) alone, and in combination, on the clonogenic potential of normal and aplastic anemia (AA) bone marrow mononuclear cells (BMMC) and CD34+ cells. AA BMMC consistently produced a significantly lower absolute number of colonies than normal, but, when account was taken of the reduced proportion of CD34+ cells in AA BM, there was no significant difference in terms of cloning efficiency (CE). However, when removed from the influence of accessory cells, the CE of AA CD34+ cells decreased significantly more than normal, indicating a defect in their function, either in terms of dependence on accessory cell-derived factors or susceptibility to cell damage when sorted. Of the factors studied, G-CSF had the most significant effect on the response of CD34+ cells from both groups when removed from their accessory cells. This was particularly true for AA CD34+ cells, whose response to cytokine stimuli containing G-CSF enabled them to match the response of normal CD34+ cells.

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STUDIES OF APLASTIC anemia (AA) bone marrow (BM) in long-term BM culture (LTBMC) have provided evidence for a stem cell defect in AA by showing an absence of or reduction in the generation of hematopoietic progenitor cells, despite normal stroma formation.1-4 Cross-over experiments, involving the recharge of irradiated pre-formed stroma with a second marrow, have permitted the separate analysis of stromal and hematopoietic stem cell function and, in two such studies, a stem cell defect has again been implicated in AA.5-7 Other evidence for an intrinsic stem cell defect has come from the isolation of CD34+ marrow cells from patients with AA using immunomagnetic beads and fluorescence activated cell sorting (FACS). These cells have been found to be reduced in number and to generate fewer colonies than normal in clonogenic culture.6,7 AA CD34+ cells grown in LTBMC on normal stroma show an absence in the generation of progenitor cells, implying a deficiency in long-term culture initiating cells (LTCIC) believed to be of the phenotype CD34+CD33-.8 Using immunophenotyping, we have previously shown that the CD34+CD33- subset is reduced in AA BM, although the clonogenic potential of these cells has yet to be determined.9

Hematopoiesis involves a cascade of differentiation steps that is driven and regulated by a group of glycoproteins known as hematopoietic cytokines.10-11 Through multiple interactions, both negative and positive, they regulate the proliferation and differentiation of hematopoietic stem cells.12

In clinical trials conducted on AA patients using granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3), no long-lasting effect on BM function has been displayed.11,13-17 Generally, patients show a transient increase in peripheral granulocyte counts, but those with no residual marrow function rarely benefit.16

The effects of cytokines on AA BM has been studied in vitro. In clonogenic culture, colony formation from AA BM is consistently low. Bagnara et al16 investigated the effect of stem cell factor (SCF) on BM from AA patients and found a modest improvement in colony growth when SCF was added in combination with GM-CSF, IL-3, and erythropoietin (Epo). In similar studies, Wodnar-Filipowicz et al9 found that, in the majority of cases, SCF synergized with GM-CSF, IL-3, G-CSF, and Epo to stimulate colony formation to up to 30% of normal values and Amann et al10 found that, in combination with Epo, SCF, but not IL-3, stimulated colony formation to up to 45% of normal values in 11 of 19 cases. Maciejewski et al17 investigated the clonogenic potential of 41 acute, untreated AA patients (26 severe and 15 moderate) and 40 hematologically recovered patients by assaying BM mononuclear cells (BMMC) and FACS-enriched CD34+ cells in a clonogenic culture system using IL-3, GM-CSF, SCF, and Epo. The acute AA samples were found to have reduced clonogenic potential (BMMC and purified CD34+ cells), whereas purified CD34+ cells from the hematologically recovered AA patients showed an increased clonogenic potential, compared with the acute patients, and their unsorted BMMC displayed normal clonogenic efficiency.1 In a study of 26 AA patients with a range of disease severity, we have shown that SCF synergized with GM-CSF, IL-3, and Epo in clonogenic culture to stimulate colony formation, in some cases to within the normal range.18 Response to SCF correlated with disease severity, with those showing the greatest response tending to be less severe, perhaps indicating that a normal response is possible, given the correct cytokine stimulation, if enough progenitor cells are present.

In the present study, we investigated the effects of GM-CSF, IL-3, G-CSF, SCF, and IL-6, alone and in combination, on the colony formation of BMMC and purified CD34+ cells from normal and AA BM. The aims of this study were as follows. (1) We wanted to investigate the effects of various cytokine stimuli on cloning efficiency (the proportion of CD34+ cells giving rise to colonies in clonogenic culture)
Patients were grouped according to severity or response at the time of study. Severity defined by Camitta et al.23 Figures given are the median with the range in parentheses. Platelet count and Hb are only given for TI patients.

Abbreviations: VS, very severe; S, severe; NS, nonsevere; NA, not applicable; PRTD, partial response (transfusion dependent); PRTI, partial response (transfusion independent); CR, complete response.

* One S and one NS patient had evidence of paroxysmal nocturnal hemoglobinuria (PNH).
† Patient with neutrophil count of 7.2 \times 10^9/L was on G-CSF therapy at the time of the study.
‡ Three patients spontaneously recovered, including 1 patient with evidence of PNH.

and to determine which give the greatest response. (2) We wanted to assess the effect of the presence of accessory cells on the cloning efficiency of BMMC, compared with purified CD34+ cells. (3) Having previously shown that the number of hematopoietic progenitors in AA BM is reduced,6 we wanted to investigate whether this reduced population is also dysfunctional and whether AA BM responds to various cytokine stimuli in the same way as normal BM to clarify whether a functional, as well as quantitative, defect is implicated in the disease.

MATERIALS AND METHODS

Normal BM specimens. Normal BM cells (n = 14) were obtained from iliac crest marrow aspirates of hematologically normal donors after informed consent. Marrow aspirates were diluted 1:1 in Iscove's Modification of Dulbecco's medium (IMDM; GIBCO, Paisley, Scotland), supplemented with 100 IU/mL penicillin-streptomycin (GIBCO) and 10 IU/mL preservative-free heparin (Leo Laboratories, Ltd, Prince's Risborough, Buckinghamshire, UK), and then centrifuged on Ficoll-hypaque (Pharmacia, St Albans, Hertfordshire, UK) to obtain the BMMC, which were washed twice in the above medium.

AA BM specimens. BM was obtained from patients with AA (n = 25; 23 patients, 2 on 2 separate occasions) referred to St George's Hospital, with the diagnosis being made on the basis of strict criteria.22 The severity of the disease was made according to the criteria of Camitta et al.23 The BMMC were separated as above. The clinical status of the AA patients is shown in Table 1.

Staining for CD34+ cells. BMMC were washed twice in phosphate-buffered saline (PBS; Sigma, Poole, Dorset, UK) supplemented with 1% fetal calf serum (FCS). The cells were then sequentially incubated with (1) human γ-globulins (30 μg/mL BMMC; Sigma) for 10 minutes at 4°C; (2) anti-CD34 antibody (10 μL/mL BMMC; QBEND 10; Immunotech S.A., Marseille, France) for 30 minutes at 4°C, followed by two washes in PBS, supplemented as above; and (3) fluorescein isothiocyanate (FITC)-conjugated rabbit F(ab)2 antiserum (6 μL/10^6 BMMC; Dako Ltd, High Wycombe, Buckinghamshire, UK) for 30 minutes at 4°C, followed by two washes, as above. The cells were then resuspended at a concentration of 10^6/mL in PBS, supplemented as above and kept at 4°C, to be ready for FACS sorting. A negative control was set up in parallel for each sample, omitting anti-CD34 antibody from the incubation.

FACS sorting of CD34+ cells. CD34+ BMMC were purified using a FACStar Plus cell sorter (Becton Dickinson, UK Ltd, Cowley, Oxford, UK). The whole nucleated cell population, excluding red blood cells and cell debris, was analyzed and scattergrams were generated by combining right-angle light scatter with fluorescence. Regions were drawn around clear-cut populations having low right-angle scatter and high fluorescence. Cells falling within this region were sorted firstly in Enrich mode at 5,000 to 6,000 cells/s and subsequently in Normal-R mode at 200 to 300 cells/s. Cells were sorted into IMDM supplemented with 10% FCS. The frequency of CD34+ cells was assessed both before and after FACS sorting and the purity and enrichment of the sorted population were then calculated.

Committed BM progenitor assay. Normal and AA BMMC (10^5) or CD34+ cells (1.67 × 10^5) were cultured in 1 mL IMDM supplemented with 30% FCS, 1% deionized bovine serum albumin (BSA; Sigma), 10^{-5} mol/L mercaptoethanol, and 0.9% methylcellulose (StemCell Technologies Inc, Terry Fox Laboratory, Vancouver, British Columbia, Canada), in 35-mm petri dishes. The following recombining growth factors were added either alone or in combination: 10 ng/mL GM-CSF (1.39 × 10^9 U/mg; Sandoz Pharma, Camberley, Surrey, UK), 100 ng/mL IL-3 (4.9 × 10^9 U/mg; Sandoz Pharma), 100 ng/mL SCF (10^9 U/mg; Immunex Corp, Seattle, WA), 100 ng/mL G-CSF (10^9 U/mg; Amgen UK Ltd, Cambridge, UK), and 100 ng/mL IL-6 (5.2 × 10^9 U/mg; Sandoz Pharma). All growth factors were aglycosylated products made in Escherichia coli, with the exception of SCF, which was glycosylated and made in yeast. Cultures were also set up in the absence of growth factors. All reagents were pretested for their ability to support optimal growth. The following cytokine combinations were studied: (1) control (no cytokines); (2) IL-6; (3) SCF; (4) GM-CSF; (5) IL-3; (6) G-CSF; (7) IL-3 + IL-6; (8) IL-3 + SCF; (9) IL-3 + GM-CSF; (10) IL-3 + G-CSF; (11) IL-3 + GM-CSF + IL-6; (12) IL-3 + SCF + IL-6; (13) IL-3 + GM-CSF + SCF; (14) IL-3 + GM-CSF + G-CSF; (15) IL-3 + SCF + G-CSF; (16) IL-3 + GM-CSF + SCF + IL-6; (17) IL-3 + SCF + IL-6 + G-CSF; (18) IL-3 + GM-CSF + SCF + G-CSF; (19) IL-3 + GM-CSF + SCF + G-CSF + IL-6.

 Cultures were set up in duplicate and incubated at 37°C in 5% CO2/95% air. Epo at 2 U/mL (Eprex; Cilag Ltd, High Wycombe, Buckinghamshire, UK) was added to all cultures on day 3. Colony-forming unit–granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-granulocyte, erythroid, monocyte (CFU-GEM) were counted on day 14 and their counts were pooled.
and expressed as either (1) the absolute colony number/10^5 BMMC
or CD34+ cells or (2) the cloning efficiency (CE), where CE = (no.
of colonies/10^5 cells)/(no. of CD34+ cells/10^5 cells).

Statistical analysis. Scatter diagrams of colony numbers and
CEs, one for each combination of environment (unsorted and sorted)
and group (AA and normal) across the 19 cytokine stimuli, were
produced to assess the data visually (data not shown). The initial
levels of CD34+ cells were also plotted to compare the two groups
in unsorted and sorted samples (data not shown). As a result of the
visual assessment and consideration of the assumptions required for
analysis of variance, the arcsine root transformation was applied to
the CEs before the analyses.

Unsorted and sorted data were analyzed separately, using split-
unit analysis of variance (ANOVA; two factors: group and cytokine
stimulus). Where appropriate, adjustments for nonsphericity were
applied.

Multiple comparisons of the mean responses to the cytokine stim-
uli were performed using the Bonferroni inequality to control for
type I error rates.

Differences between normal and AA responses to specific cyto-
kine stimuli were analyzed using unpaired t-tests, if the assumptions
for this test were verified, or Wilcoxon tests, if not.

The effect of each cytokine combination on unsorted and sorted
BM for each sample group was analyzed using a within-subject
ANOVA (two factors: environment and cytokine stimulus).

Differences in response between unsorted and sorted samples
(normal and AA separately) were analyzed using paired t-tests or
Wilcoxon tests.

RESULTS

The number of colonies produced increased as the cyto-
kine stimulus moved from one factor to two, three, etc, for
both normal and AA cells, whether sorted or unsorted. Figure
1 shows the mean absolute number of colonies, with 95%
confidence intervals, produced by normal and AA BM in
response to the 19 cytokine combinations, for unsorted and
sorted BM, respectively. Figure 1A shows the much higher
response of normal, compared with AA, BM to the 19 cyto-
kine combinations. Figure 1B shows that, when sorted, the
response of AA BM is much closer to, although still lower
than, that of normal BM. For many combinations, the 95%
confidence intervals of the means of the two groups overlap.
These results are obviously dependent on the initial level of
CD34+ cells in the samples. Mann Whitney U tests showed
a significant difference between normal (mean, 2.16% ±
0.33%; range, 0.9% to 6.0%) and AA (mean, 0.65% ±
0.068%; range, 0.2% to 1.5%) CD34+ levels before sorting
(P < .00002). After sorting, there was no significant differ-
ence between normal (mean, 88.86% ± 1.35%; range, 80%
to 95%) and AA (mean, 83.56% ± 3.10%; range, 20% to
97% [only 1/25 sorted AA samples had a purity <62%])
CD34+ levels (P = .3311). To control for CD34+ levels
when comparing their functional response to cytokine stim-
uli, results were expressed in terms of CE. Figure 2 shows
the mean transformed CEs, with 95% confidence intervals,
of normal and AA BM, in response to the 19 cytokine combi-
nations, for unsorted and sorted BM, respectively. It shows
that, for both normal and AA, CE is higher in unsorted than
in sorted BM. In the unsorted samples, the AA means
are much closer to those of the normal samples than in the sorted
samples.

Statistical analysis of unsorted BM. Results of the group
by cytokine stimulus ANOVA showed that the interaction
is insignificant (P = .1448). A series of two sample t-tests
comparing the responses in the two groups for each cytokine
stimulus showed very insignificant results, indicating no dif-
fferences between the groups for any cytokine stimulus, there-
fore giving no interaction. Moreover, the overall group effect
was insignificant (P = .3306); there was no significant differ-
ence between the groups in terms of CE, irrespective of
cytokine stimulus (Fig 2A). Therefore, the CE of unsorted
AA BM is not significantly different from normal.

However, the cytokine stimulus effect was highly signifi-
cant (P = .0001). One or more cytokine stimuli are, there-
fore, significantly different from one or more others. To
establish which cytokine combinations are significantly dif-
ferent from which, the normal and AA samples were tested
Statistical analysis of sorted BM. Results of the environment by cytokine stimulus ANOVA performed on each sample group separately showed that, for both normal and AA, the main effects of cytokine stimulus (normal, $P = .0001$; AA, $P = .0001$) and environment (normal, $P = .008$; AA, $P = .0001$) and the cytokine stimulus by environment interaction (normal, $P = .0001$; AA, $P = .0001$) were all highly significant. To clarify the cause of significance, each stimulus was selected and the response in the two environments were compared. Paired $t$-tests and Wilcoxon tests were used to compare the two groups. If the level of significance is again taken as $P = .01$, the results show that, for both normal and AA BM, the unsorted and sorted samples are not significantly different for stimuli 10, 14, 15, 17, 18, and 19. Also, in the normal subjects, stimulus 6 showed a nonsignificant difference between the two environments (Fig 3).

DISCUSSION

We investigated the effects of a wide range of cytokine stimuli on the clonogenic potential of normal and AA BMMC and purified CD34$^+$ cells.

(1) It was found that the clonogenic potential of BM is greatly influenced by the effects of the exogenous cytokines studied. For the unsorted samples, there was no significant difference between normal and AA BM in terms of CE, irrespective of cytokine stimulus (Fig 2A). The multiple comparison of mean responses to cytokine stimuli, combining normal and AA data, showed no clear pattern emerging as to which cytokine stimuli stand out in their effect on CE but only that, generally, the more cytokines present, the greater the CE. The cytokine production of accessory cells presumably providing a plethora of others. We sorted CD34$^+$ cells to remove them from this influence, laying bare their functional response to the cytokine stimuli administered in an attempt to highlight differences in this response between normal and AA.

(2) On doing so, our first finding was that, for both normal and AA BM, CE decreases after sorting (Fig 3). For each cytokine stimulus studied, the CE of sorted BM was lower than that of unsorted BM. This is to be expected, given that the maximum stimulus contained only five cytokines, with accessory cells presumably providing a plethora of others. However, there were several combinations of cytokines that, although producing a lower response in the sorted than in the unsorted samples, did not produce a significantly lower response; for both normal and AA BM, these were 10, 14, 15, 17, 18, and 19 (Fig 3). These cytokine stimuli comprise all the 2-, 3-, 4-, and 5-factor combinations containing G-CSF. In addition, for normal subjects, G-CSF alone (no.
the two groups to a level where they no longer differed significantly and to a purity that enabled us to investigate and compare their clonogenic potential.

Unsorted, AA BM produced a significantly lower absolute number of colonies than did normal BM, regardless of the cytokine stimulus (Fig 1A). However, this difference would appear to be a reflection of the reduced level of CD34+ cells in AA BM, because, in terms of CE, there was no significant difference between normal and AA BM. For some cytokine combinations, in fact, AA CE was equal to, or higher than, normal CE (Fig 2A). One might argue from these findings that the CD34+ cells of these AA patients were not dysfunctional, with the reduced number of colonies produced by their BM simply being a result of their reduced numbers of CD34+ cells. However, it is possible that a dysfunction in AA CD34+ cells could be masked by the influence of compensating accessory cells. Using FACS, we removed CD34+ cells from the influence of accessory cells, increasing their clonogenic dependence on the administered exogenous cytokine stimuli.

Although the CE of both normal and AA BM decreased significantly when sorted, the CE of AA BM decreased significantly more than did that of normal BM (Fig 2B). Not only do AA CD34+ cells have a lower CE than normal after sorting, but their pattern of response to the cytokine combinations also differs from normal. This could be explained in two ways: (1) the mechanics of cell sorting might in some way damage CD34+ cells, perhaps by decreasing cytokine receptor expression, thereby lowering CE (AA CD34+ cells may be more "fragile," more susceptible to this kind of damage); or, (2) as suggested above, AA CD34+ cells might be more dependent on the stimulation and control of accessory cells than normal CD34+ cells. In either case, it appears that AA CD34+ cells differ from normal CD34+ cells and are in some way dysfunctional. This was examined further in a series of two-sample Wilcoxon and t-tests, which showed that normal and AA sorted samples are clearly significantly different for stimuli 4, 5, 7, 9, 11, 15, and 19, but not significantly different for 6, 10, 13, and 14 and 15 (Fig 2B). G-CSF again seems to play a key role here, because it is present in 6, 10, 14, and 15, implying that it has an equally powerful effect on the CE of AA CD34+ cells as it does on normal CD34+ cells. The fact that stimulus 19 contains G-CSF but does not stimulate normal and AA CD34+ cells equally may not be contradictory, because this is the combination containing the most cytokines and the effect of G-CSF is, therefore, less noticeable. IL-3 + GM-CSF + SCF (no. 13) also appears to stimulate both populations equally.

To establish which cytokine combinations are significantly different from which, for the normal and AA samples separately, multiple comparisons of the mean responses were conducted. Generally, normal CD34+ cells showed a much wider range of response to the stimuli than AA CD34+ cells, implying that they are more responsive to the synergistic effects of the cytokines. However, there were some instances in which the AA CD34+ samples show significant differences, whereas the normal CD34+ cells do not. Thus, for AA, but not normal, (1) the effect of G-CSF is significantly greater than that of IL-3 + IL-6, IL-3 + GM-CSF, and IL-3 + GM-CSF + IL-6 and (2) the effect of IL-3 + GM-CSF...
+ SCF is significantly greater than that of both IL-3 + IL-6 and IL-3 + GM-CSF. It is clear from Fig 2B that, of the five cytokines studied, G-CSF has by far the greatest overall effect on AA, as well as normal, CD34+ cells. Moreover, in contrast to normal, IL-3 seems to have little effect on AA CD34+ cells, either alone or in combination with IL-6 and/or GM-CSF. However, in combination with GM-CSF + SCF (stimulus no. 13), IL-3 does have an effect; this was the only cytokine combination not containing G-CSF that stimulated AA CD34+ cells to the same extent as normal CD34+ cells (see above).

As already stated, in agreement with other groups, we found that AA BM contains significantly less CD34+ cells than normal BM. There was a correlation between disease severity and proportion of CD34+ cells: all 3 severe AA patients had levels less than the mean (0.65% ± 0.068%); of those with complete response (CR), 7 of 11 had levels greater than the mean, whereas the remaining 4 were just below it (2 with 0.6% and 2 with 0.5%). Of the 25 samples analyzed, 8 were from patients who had received no previous therapy, including 3 on presentation (2 nonsevere and 1 severe; Table 1). There was also a degree of correlation between disease severity and response to cytokine stimuli, in agreement with previous work.7 All 3 severe AA samples fell below the normal CE 95% confidence interval range, both before and after sorting. Indeed, 2 of 3 did not show any response, regardless of cytokine stimulus. Of the 9 samples falling within the normal range, 6 were in CR and 3 were nonsevere. Only 1 in CR was out of the normal range before and after sorting. However, there were 4 in CR and 2 in partial response (PR) that, although falling within the normal range before sorting, were outside the normal range after sorting. This may support the previously reported abnormal in vitro growth of BM from patients who have responded to treatment.2 The remaining samples (2 nonsevere and 3 in PR) tended to fall outside the normal range both before and after sorting. In a few cases, insufficient numbers of cells were obtained after sorting to allow the analysis of response to all cytokine combinations.

In summary, we found that the reduced CD34+ compartment of AA BM is, in addition, dysfunctional. G-CSF appears to compensate for this dysfunction to some extent and there may be other compensating factors produced by accessory cells, resulting in the insignificant difference between normal and AA CE before sorting. This dependence on accessory cells may account for the significant decrease in AA CE seen after sorting, which may also be partly, or wholly, attributable to the fragility/instability of AA CD34+ cells compared with normal CD34+ cells.

G-CSF makes a significant contribution to the response of CD34+ cells from both groups when removed from the influence of accessory cells. This seems particularly true of AA CD34+ cells, because it enables them to match the response of normal CD34+ cells when sorted. A recent report suggests that the administration of G-CSF with ALG and steroids in the treatment of SAA patients improves their response rate and survival; our data may support the hypothesis that this is due to the mobilizing effect of G-CSF on early progenitors.28 Our data also provide evidence that AA CD34+ cells may be dysfunctional in their response to IL-3. There appears to be a correlation between disease severity not only with the proportion of CD34+ cells in BM, but also with the dysfunctionality of these cells, lending further support for a stem cell functional defect in this disease.

Whether this dysfunctionality is due to an intrinsic abnormality or to a difference in the functional state of the AA CD34+ cells is unclear, although the corrective effect of G-CSF may support the latter. The question of whether the observed dysfunctionality applies to the whole stem cell compartment or to a specific subpopulation will be addressed in assays for LTCIC and single-cell cultures.

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