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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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,9 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, Princes 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/106 BMMC; Sigma) for 10 minutes at 4°C; (2) anti-CD34 antibody (10 μL/106 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 antimouse IgG (6 μL/106 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 105/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 FACSStar 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 Enhance 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 (105) or CD34+ cells (1.67 × 105) were cultured in 1 mL IMDM supplemented with 30% FCS, 1% deionized bovine serum albumin (BSA, Sigma), 10−8 mol/L mercaptoethanol, and 0.9% methylcellulose (StemCell Technologies Inc, Terry Fox Laboratory, Vancouver, British Columbia, Canada), in 35-mm petri dishes. The following recombinant growth factors were added either alone or in combination: 10 ng/mL GM-CSF (1.39 × 109 U/mg; Sandoz Pharma, Camberley, Surrey, UK), 100 ng/mL IL-3 (4.9 × 107 U/mg; Sandoz Pharma), 100 ng/mL SCF (107 U/mg; Immunex Corp, Seattle, WA), 100 ng/mL G-CSF (108 U/mg; Amgen UK Ltd, Cambridge, UK), and 100 ng/mL IL-6 (5.2 × 107 U/mg; Sandoz Pharma). All growth factors were aglycosolated 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-3; (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 stimuli were performed using the Bonferroni inequality to control for type I error rates.

Differences between normal and AA responses to specific cytokine 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 cytokine 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 cytokine 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 difference 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 stimuli, 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 combinations, 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 differences between the groups for any cytokine stimulus, therefore giving no interaction. Moreover, the overall group effect was insignificant (P = .3306); there was no significant difference 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 significant (P = .0001). One or more cytokine stimuli are, therefore, significantly different from one or more others. To establish which cytokine combinations are significantly different from which, the normal and AA samples were tested...
for heterogeneity of variance before being collapsed into a single group, after which a multiple comparison of the mean responses to the cytokine stimuli was performed using the Bonferroni inequality to control overall type I error rates (data not shown; see Discussion).

**Statistical analysis of sorted BM.** Results of the group by cytokine stimulus ANOVA showed that the interaction is significant ($P = .0148$). Therefore, some cytokine combinations produce significantly different effects in the two groups. The group main effect is also highly significant ($P = .0069$). After sorting, therefore, the CE of AA CD34$^+$ cells was statistically significantly different from normal. To establish the cause of this difference, a series of two-sample Wilcoxon and $t$-tests of the transformed data were conducted for each cytokine combination. Given the number of tests, if $P = .01$ is taken as the level of significance, stimuli 4, 5, 7, 9, 11, 16, and 19 were clearly significantly different, whereas 6, 10, 13, 14, and 15 were clearly not (Fig 2B). The cytokine stimulus main effect is highly significant ($P = .0001$). To establish which combinations are significantly different from which for the normal and AA samples separately, further subject by cytokine stimulus ANOVAs and Bonferroni multiple comparisons were performed (data not shown; see Discussion).

Statistical comparison of unsorted and 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 clearly plays a key role in substituting for the effects of cytokines produced by these cells; G-CSF is, therefore, less noticeable. IL-3, GM-CSF, and IL-6, IL-3, IL-6, and IL-6, IL-3, GM-CSF, and IL-3, GM-CSF, and IL-6 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 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, 16, and 19, but not significantly different for 6, 10, 13, 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

6) produced a nonsignificant difference between the two environments.

The effect of G-CSF could be interpreted in two ways: either G-CSF is a major component of the cytokine stimulus of accessory cells or its effects overlap with those of other cytokines produced by these cells; G-CSF is an early as well as late acting cytokine24-26 and is known to be produced by LTBMSC stromal cells27. In either case, exogenous G-CSF clearly plays a key role in substituting for the effects of accessory cells in stimulating the CE of purified normal and AA CD34+ populations.

(3) In agreement with previous studies, we found that AA BM contains significantly less CD34+ cells than normal BM. Using FACS, we enriched the CD34+ cells in BM from 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.
+ 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 AA 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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