Quantitative Analysis of Cobblestone Area-Forming Cells in Bone Marrow of Patients With Aplastic Anemia by Limiting Dilution Assay

By Hubert Schrezenmeier, Marianne Jenal, Friedhelm Herrmann, Hermann Heimpel, and Aruna Raghavachar

In the past, the analysis of primitive human hematopoietic progenitor cells with repopulating activity was limited by lack of appropriate in vitro assay systems. It was recently shown that cobblestone area-forming cells (CAFC) giving rise to cobblestone areas after 5 weeks in long-term marrow cultures (LTMC) represent a population of pluripotent progenitor cells with long-term marrow-repopulating activity. We have used a microtiter limiting-dilution-type human LTMC system to quantify the frequency of CAFC (week 5) in aplastic anemia (AA). In bone marrow mononuclear cells (BM-MNC) of healthy donors (n = 36) we observed a mean frequency of 84.4 CAFC per 10^5 BM-MNC (95% confidence interval limits, 66.4 to 102.4). The mean frequency of CAFC in BM of 31 AA patients was 6.6 per 10^5 BM-MNC (95% confidence interval limits, 5.3 to 7.9; n = 47). This frequency is significantly lower as compared with controls (P < .0001).

SEVERAL PATHOGENETIC mechanisms have been proposed to account for the bone marrow (BM) failure in aplastic anemia (AA). These include an intrinsic defect of hematopoietic stem cells, lymphocyte-mediated suppression of hematopoiesis, dysfunction of the BM microenvironment, and abnormalities in regulation of hematopoiesis by humoral factors. Several studies have shown that AA patients who responded to immunosuppressive treatment are at high risk for relapse of aplasia or for the evolution to clonal disorders of hematopoiesis. These clinical observations suggest that patients in remission are not cured of their disease but that some pathophysiologic conditions might have persisted in responders after immunosuppressive treatment.

Short-term cultures have shown a significant reduction in all types of committed hematopoietic progenitor cells. This defect does not normalize after response to immunosuppressive treatment. A hematopoietic defect is also demonstrable in long-term marrow cultures (LTMC) of AA marrow. Compared with controls, the number of colony-forming units–granulocyte-macrophage (CFU-GM) produced in LTMC of AA marrow is significantly lower and CFU-GM are detected in LTMC for a shorter period of time. How LTMC of AA marrow is significantly lower and CFU-GM produced in LTMC correlates with long-term repopulating ability. A hematopoietic defect is also demonstrable in long-term marrow cultures (LTMC) of AA marrow. Compared with controls, the number of colony-forming units–granulocyte-macrophage (CFU-GM) produced in LTMC of AA marrow is significantly lower and CFU-GM are detected in LTMC for a shorter period of time. However, neither the measurement of committed progenitors nor the analysis of growth in standard-type LTMC allows the enumeration of very primitive hematopoietic cells. Combination of LTMC with limiting dilution method makes a quantitative assessment of primitive cells feasible. Sutherland et al have reported a method to assess the frequency of LTMC-initiating cells that give rise to clonogenic cells for at least 5 weeks of culture. This technique requires replating of individual wells. In contrast, the assay for limiting dilution analysis of cobblestone area-forming cells (CAFC) described by Ploemacher et al allows a direct visual evaluation without replating. It has been shown that the frequency of precursors forming cobblestone areas on day 28 or 35 of LTMC correlates with long-term repopulating ability.

We have used this limiting dilution analysis of CAFC for quantitative assessment of the hematopoietic defect in AA at the level of very primitive progenitor cells. Furthermore, we have examined whether a persisting defect of the CAFC can be shown also after autologous hematologic reconstitution in patients who had received previous immunosuppressive treatment.

PATIENTS AND METHODS

Patients

A total of 31 patients with AA was studied. The characteristics of the AA patients are summarized in Table 1. The first analysis of CAFC was performed at diagnosis before the first immunosuppressive treatment in 11 patients. Twenty patients were analyzed for the first time after immunosuppressive treatment, with a median follow-up time of 12 months (range, 0 to 216 months) after diagnosis. Thirteen patients (nos. 2, 4, 11, 12, 15, 19, 24, 25, 26, 27, 28, 30, and 31) were investigated at two or three occasions. Five of these patients (nos. 11, 12, 15, 19, and 26) were examined both at diagnosis and after having reached remission. Thirty-three CAFC measurements were performed when the patients were still pancytopenic, whereas 14 assessments were performed in patients who had achieved at least a partial response to immunosuppressive treatment. The diagnosis of AA was established according to the criteria of the International Agranulocytosis and Aplastic Anemia Study Group. AA was considered as severe if it met the following criteria: hypocellular BM and a cytopenia in peripheral blood with at least two of the following criteria: a granulocyte count of less than 0.5 × 10^3/L, a platelet count of less than 20 × 10^3/L, and a reticulocyte count of less than 20 × 10^3/L. Patients fulfilling these criteria but having a granulocyte count of less than 0.2 × 10^3/L were classified as very severe AA (vSAA). Patients with AA who did not meet the criteria for severe disease were classified as nonsevere. Patients received immunosuppressive ther-

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apy as outlined in Table 1: antilymphocyte globulin (ALG; Lymphoglobulin Merieux; Institut Merieux, Leimen, Germany) at 0.75 mL/kg subcutaneously daily until day 112. Monoclonal antibody against the interleukin-2 receptor (IL-2R MoAb; Neupogen, Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563; cyclosporin A (CyA; Sandimmun; Sandox, Nürnberg, Germany) at 5 to 12 mg/kg orally initially, with dose adjustment according to blood trough levels for 3 months.26-28 Granulocyte colony-stimulating factor (G-CSF; Neupogen; Amgen, Thousand Oaks, CA) was administered at 5 μg/kg subcutaneously daily until day 112. Monoclonal antibody directed against the interleukin-2 receptor (IL-2R MoAb; BTK563;
Biotest, Dreieich, Germany) was administered IV at a dose of 10 mg on days 1 through 3 and at a dose of 5 mg on days 4 through 10, 12, 14, 16, 18, and 20. The criteria for evaluation of response to immunosuppression were as follows. A complete remission (CR) was determined by a hemoglobin level greater than 12.0 g/dL, a neutrophil count greater than 1.5 × 10^9/L, and a platelet count greater than 100 × 10^9/L. A partial remission (PR) was indicated by complete resolution of transfusion requirements and an increase above pretreatment levels of at least 0.5 × 10^9/L for neutrophils, 30 × 10^9/L for platelets, and 30 × 10^9/L for reticulocytes. No response (NR) means persistent pancytopenia with no change of status or death. The overall response rate is defined as the sum of complete and partial responses 3 months after the initiation of therapy.

**Preparation of Cells**

After informed consent was obtained, BM from patients with AA and healthy controls was collected by aspiration from the posterior iliac crest. BM mononuclear cells (BM-MNC) were separated on Lymphoprep (1.077 g/mL, Nycomed, Oslo, Norway). BM-MNC were washed and resuspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Eggenstein, Germany) with 20% (vol/vol) preselected fetal calf serum (FCS; GIBCO). In IMDM supplemented with 0.1 mol/L hydrocortisone sodium succinate (Sigma, Munich, Germany) in 6-well tissue culture plates (Nunc). BM cells from all donors formed a confluent stroma and a characteristic red appearance on days 7 and 14 of culture. CFU-E (erythroid colonies) were identified by their preselected fetal calf serum (FCS; GIBCO), erythroid colony-forming units-erythroid (BFU-E). Erythroid colonies were identified by their characteristic red appearance on days 7 and 14 of culture. CFU-E (1 to 2 erythroid clusters) were scored on day 7 and BFU-E (>3 erythroid clusters) were scored on day 14 of culture. All cultures were set up in triplicate in 96-well flat-bottomed plates (Nunc, Nürtingen, Germany) and were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

**Colonies Assays**

For analysis of CFU-GM, the BM-MNC (equivalent to 2 × 10⁶ cells/culture well) were plated into a layer composed of 0.3% agar, and healthy controls was collected by aspiration from the posterior iliac crest. BM mononuclear cells (BM-MNC) were separated on Lymphoprep (1.077 g/mL, Nycomed, Oslo, Norway). BM-MNC were washed and resuspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Eggenstein, Germany) with 20% (vol/vol) preselected fetal calf serum (FCS; GIBCO), and were incubated in 0.1 mL LTMC medium per well in 96-well flat-bottomed microwells (Nunc). The adherent layer of cells had been established in the microplates. The number of cobblestone areas per culture (day 35) are plotted against the number of input cells seeded on the stromal cell layer for a representative experiment with BM-MNC of a healthy donor (○) and a patient with AA (■). The slope of the linear regression of the logarithm of the values is 1.42 (healthy donor) and 1.23 (AA). The probability that the slope is significantly different than 0 is P = .0001 (control) and P = .0008 (AA).

**RESULTS**

BM-MNC of controls and AA patients were overlaid on preformed irradiated allogeneic stroma layers. The allogeneic stroma layers were capable of supporting the formation of cobblestone areas. First, the relationship between the number of BM-MNC that were initially overlaid on the stroma layer and the number of cobblestone areas present after 35 days of culture was investigated. Figure 1 shows a representative experiment with BM-MNC from a control and a patient with AA. The number of cobblestone areas per culture (day 35) are plotted against the input cell number. In both AA patients and controls, the number of cobblestone areas per culture was linearly related to the input cell number. The slopes of the regression line of log (no. of cobblestone areas) versus log (input cells) were not different among samples from controls and AA patients (P > .05).

The frequency of progenitors giving rise to a cobblestone area after 35 days of culture was determined in limiting dilution experiments. Examples of limiting dilution experiments comparing the frequency of CAFC in AA and control BM-MNC are shown in Fig 2. Assuming a single-hit model, the frequency of CAFC was calculated using the maximum likelihood method with iterative approximations as described by Taswell.30

In BM of 36 healthy controls, the mean absolute number of CAFC was 84.4 per 10⁶ BM-MNC (95% confidence interval [CI] limit, 66.4 to 102.4). In 47 CAFC assays performed...
with BM-MNC of 31 AA patients, the mean frequency of CAFC was significantly reduced to 6.6 CAFC per 10^5 BM-MNC (95% CI limit, 5.3 to 7.9; P < .0001 compared with healthy controls). CAFC were significantly reduced compared with controls both in BM of cytopenic AA patients and AA patients in remission after treatment. The absolute number of CAFC per 10^5 BM-MNC of controls and AA patients is depicted in Fig 3. In pancytopenic AA patients, the mean frequency of CAFC was 6.2 per 10^5 BM-MNC (95% CI limit, 4.6 to 7.8; P < .0001, compared with healthy controls). In 13 patients who were analyzed after they had achieved at least a partial response to immunosuppressive treatment, the mean frequency of CAFC was still significantly lower than in controls (7.6 per 10^5 BM-MNC; 95% CI limit, 4.9 to 10.4; P < .0001 compared with controls).

The difference in CAFC numbers between aplastic AA patients and AA patients in remission after treatment is not statistically significant (P = .1). The absolute CAFC numbers of 13 patients who were analyzed at several occasions during the course of the disease are shown in Fig 4. It is shown that patients with low CAFC numbers at diagnosis continue to have low CAFC after immunosuppression even if they had entered remission.

The number of CAFC in BM did not reflect the severity of the aplasia. There was no correlation of CAFC number and severity of the disease or the remission status at time of CAFC analysis (Table 2). Furthermore, there was no correlation between the number of CAFC and the response to immunosuppressive treatment (P = .58; data not shown). The reduction in the frequency of CAFC did not correlate with the duration of aplasia (coefficient of correlation, .12; P = .42).

The mean frequencies of BM CFU-GM, BFU-E, and CFU-E in controls and patients with AA are listed in Table 3. The number of committed progenitor cells was significantly reduced both in aplastic AA patients and in patients after response to treatment as compared with controls. In contrast to CAFC, committed progenitor cells were higher in AA patients in remission compared with patients in aplasia.

| Patients | CAFC per 10^5 BM-MNC
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<tbody>
<tr>
<td>Aplastic</td>
<td></td>
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<tr>
<td>vSAA/SAA</td>
<td>6.7 (3.4-9.9)</td>
</tr>
<tr>
<td>nSAA</td>
<td>5.9 (4.4-7.8)</td>
</tr>
<tr>
<td>Partial remission</td>
<td>6.6 (2.8-10.4)</td>
</tr>
<tr>
<td>Complete remission</td>
<td>9.2 (3.5-14.9)</td>
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* Mann-Whitney test.
† In 13 patients serial assessments were performed. The total number of CAFC assays in the 31 patients was n = 47.
The pathogenesis of AA is still unknown. Several recent reports argue against a defective microenvironment and are in favor of a stem cell defect as the cause of AA. The frequencies of CD34+, CD33+ and CD34+CD33− populations in BM of patients with AA are reduced as compared with normals. Growth of myeloid and erythroid colonies from separated AA CD34+ cells is decreased in comparison to normal CD34+ cells. Also in LTMC of AA BM, the production of nonadherent cells and CFU-GM is significantly reduced. Marsh et al have shown a defect of hematopoietic cell function with normally functioning stroma by crossover experiments inoculating nonadherent BM cells of AA patients onto preformed, irradiated normal stroma and vice versa. In these studies, the stage of differentiation at which the defect occurred remained unclear. The defect seen in LTMC might either be a consequence of absent or defective long-term culture-initiating cells (LTC-IC) or reflects an impaired differentiation of uncommitted to committed progenitor cells.

These studies indicate both a quantitative reduction of stem cells as well as a qualitative defect of early progenitor cells in AA. However, none of these studies measured quantitatively the number of CAFC or LTC-IC as an in vitro surrogate of long-term repopulating cells. Therefore, we have applied a human limiting dilution-type assay to the quantitative analysis of CAFC in AA. We inoculated BM-MNC of healthy controls or AA patients under limiting dilution conditions on preformed irradiated primary human stromal layers that were established from healthy human donors. To exclude a probable stroma effect, the limiting dilution analysis was performed on irradiated stroma layers established from normal donors. The irradiation did not affect the supportive stroma function in our system, which is in keeping with the findings of earlier reports. The use of primary human stromal layers is one of the major differences between our system and the human CAFC assay recently described by Breems et al. who used a murine preadipose stromal cell line FBMD-1. In contrast to Breems et al. who added IL-3 and G-CSF to their culture system, we did not add any exogenous growth factors to our culture system. With our limiting dilution CAFC assay, the mean CAFC frequency of 36 healthy donors was 84.4 per 10⁶ BM-MNC, as determined at week 5 of culture. Breems et al. reported a CAFC week-5 frequency of 44/10⁶ BM-MNC in 8 normal BM samples. In an LTC-IC assay, Sutherland et al. found a frequency of 37 LTC-IC per 10⁶ percolled BM cells of 5 healthy controls. The differences in the experimental systems may account for the slight discrepancy between the frequencies reported.

The frequency of CAFC in BM-MNC of 31 patients with AA was reduced about 13-fold as compared with controls. To our knowledge, this is the first quantitative assessment of the hematopoietic defect in AA at the level of CAFC. A similar reduction of primitive progenitor cells was recently reported by Maciejewski et al using the LTC-IC assay. The longitudinal studies suggest that CAFC assays are not useful for prediction of possible therapy responses. The lack of correlation of CAFC with clinical characteristics (eg, severity or duration of disease and response to treatment) is in keeping with other studies that also failed to correlate in vitro growth of AA BM cells with clinical parameters.

Our results provide direct evidence that the hematopoietic defect in AA is present at the level of CAFC. However, there remain open questions. The nature of the defect is not clarified by our study. We cannot discriminate whether the reduced frequency of CAFC is due to an intrinsic stem cell defect (eg, a mutation in a gene regulating growth and differentiation) or is the consequence of extrinsic damage (eg, immune reaction towards progenitors at the CAFC level). There are many arguments supporting the view that immunemediated suppression of hematopoiesis is an important factor in the pathogenesis of AA. Nevertheless, attempts to show direct specific cytotoxic activity of T cells against autologous progenitor cells failed in most cases. One of several possible explanations for this failure relates to the use of inappropriate target cells. In the past, inhibitory effects of lymphocytes or lymphocyte-derived cytokines were monitored at the level of committed hematopoietic progenitor cells. However, this does not exclude an immune reaction directed against very early progenitor cells. The CAFC assay system provides the experimental tool to test this possibility.

Despite normal or near normal peripheral blood cell counts after response to immunosuppressive treatment, all AA patients in our study still had a low CAFC frequency. For estimating the total number of residual primitive progenitor cells in AA, one has to consider the marrow cellularity. Low CAFC frequency (per mononuclear cell) in remission patients means that the total residual number of CAFC is higher than in aplastic patients but lower than in normal controls. This finding is in accordance with the report that LTC-IC in patients who had hematopoietic recovery after treatment remained below the normal range in the majority of patients. Marsh et al. reported that a marked defect of hematopoiesis in LTMC can be shown regardless of the degree of response to treatment. These results support the notion that most long-term survivors after immunosuppressive treatment are not really cured of their disease. Most of them exhibit persisting abnormalities, including subnormal peripheral blood counts, pathologic BM morphology, reduced frequency of CFU-GM, and reduced number of CD34+ cells.

It is not clear why AA patients achieve at least a partial response after treatment despite a persisting defect at the CAFC level. One can speculate that there are compensatory
mechanisms that lead to an expansion of more mature cells to maintain a normal or near normal peripheral blood count. This hypothesis is supported by the observation that the frequency of committed progenitors increased after response to immunosuppression, whereas frequencies of CAFC did not differ between aplastic and recovered AA patients. An alternative explanation is that in vivo stem cells are recruited into proliferation and differentiation by mechanisms that do not operate in LTMC. An overexpression of early and late-acting hematopoietic growth factors and inhibitory cytokines has been shown in AA. In vivo, all these factors might influence the proliferation of CAFC. The quantitative CAFC assay should make it possible to separately investigate the effects of these factors.

We conclude that the limiting dilution assay for CAFC permits a quantitative assessment of primitive progenitor cells in AA and other BM disorders. This assay system provides an in vitro approach to a number of relevant issues regarding the pathogenesis of AA. Effects of therapeutic agents (eg, hematopoietic growth factors) on the residual primitive progenitor cells in AA can be studied as well.

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Quantitative analysis of cobblestone area-forming cells in bone marrow of patients with aplastic anemia by limiting dilution assay

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