Inhibition of Erythroid Colony Formation by Autologous Bone Marrow Adherent Cells From Patients With the Anemia of Chronic Disease

By G. David Roodman, Victor W. Horadam, and Terry L. Wright

To determine the role marrow-adherent cells may play in the anemia of chronic diseases, marrow samples were collected from ten patients with the anemia of chronic disease, seven control patients with cancer but without the anemia of chronic disease, and five normal volunteers. Marrow was either cultured directly or first depleted of adherent cells and then cultured. Plasma clots containing $6 \times 10^6$ nonadherent marrow cells were cocultured with $6 \times 10^6$ adherent cells and then cultured. Plasma clots containing $6 \times 10^6$ nonadherent marrow cells were cocultured with marrow-adherent cells prepared by incubating $6 \times 10^6$ unfractionated marrow cells in microtiter plates and removing the nonadherent cells. Adherent cell depletion of marrow from patients with anemia of chronic disease significantly increased erythroid colony formation. Coculture of adherent cells from anemic patients with autologous nonadherent marrow cells inhibited erythroid colony formation significantly.

The anemia of chronic disease is one of the most frequently encountered anemias in adults. It is found in patients with malignancies or chronic inflammatory processes and occurs in patients without demonstrable bone marrow involvement by the malignant or infectious process. The anemia of chronic disease is characterized by a mild to moderate hypoproliferative anemia, decreased serum iron, decreased serum iron-binding capacity, and normal or increased reticuloendothelial iron stores.

The pathogenesis of the anemia of chronic disease is unclear. At least three distinct mechanisms have been proposed to contribute to the anemia. Cartwright and coworkers demonstrated a shortened red cell survival in these patients, but the shortened red cell survival was insufficient to explain the degree of anemia. Douglas et al. suggested that a defect in iron reutilization occurs in the anemia of chronic disease, resulting from a blockage of iron release from reticuloendothelial stores. They suggested that the blockage of iron release results in relative iron deficiency and was responsible for the anemia. Other investigators have either been unable to demonstrate an iron reutilization defect or have questioned the significance of such a blockage. Early studies by Ward et al. reporting low levels of erythropoietin in patients with the anemia of chronic disease, could not be confirmed by other investigators.

Many studies have demonstrated that nonerythroid cells can stimulate or suppress erythropoiesis. Lymphocytes, monocyte-macrophages, and cancer cells affect erythropoiesis in vitro. Gordon and coworkers have suggested that bone marrow macrophages normally stimulate erythroid colony (CFU-E) formation. However, bone marrow adherent cells from patients with chronic fungal infections have been reported to suppress erythroid colony formation, and this suppression was lost after antifungal therapy. In the present study, we tested the effects of bone marrow adherent cells on CFU-E and erythroid burst (BFU-E) growth in marrow samples from patients with the anemia of chronic disease, in control patients who have malignancies but without the anemia of chronic disease, and in normal volunteers using an in vitro semisolid culture assay of erythropoiesis.

MATERIALS AND METHODS

Anemic Patients

Ten patients with anemia and malignancies or chronic inflammation were studied. All patients had decreased serum iron and total iron-binding capacity, normal or increased iron stores on bone marrow examination, reticulocytopenia, and the absence of renal dysfunction (serum creatinine less than 1.0 mg/dl or normal creatinine clearance). The characteristics of the patients are shown in Table 1. This group will be referred to as "anemic patients."

Control Patients

The control patients included seven male cancer patients undergoing bone marrow harvest for autologous marrow transplantation. All
patients in this group had hematocrits greater than 35% (mean hematocrit 42±2%) with no evidence of reticulocytosis. None of the patients with malignancies had evidence of bone marrow involvement in the marrow biopsies or had received chemotherapeutic agents within the preceding 2 mo. This group will be referred to as "control patients."

Normal Volunteers

Normal volunteers consisted of five healthy paid donors. This group will be referred to as "normal patients." The protocol for this study was approved by the Human Research Review Board at the University of Texas. Informed consent was obtained from all subjects.

Bone Marrow Samples

Bone marrow samples (2–5 ml) were aspirated from the posterior superior iliac crest under xylocaine anesthesia into syringes containing 1,000 U/ml of preservative-free heparin in alpha-Minimal Essential Medium (α-MEM). Marrow samples were diluted with 2 vol of α-MEM, and marrow mononuclear cells were recovered after density gradient centrifugation over Hypaque-Ficoll. Cells at the interface were collected, washed 3 times with α-MEM, and nucleated cells counted in a hemocytometer. The cells were resuspended in α-MEM-20% fetal calf serum (FCS) at 4–6 × 10^6 nucleated cells/ml. An aliquot of the marrow mononuclear cells was kept on ice until cultured, a period of less than 2 hr in all studies.

The remaining marrow mononuclear cells were used for studies involving nonadherent marrow cells. An aliquot of the marrow cell suspension was diluted either 10-fold, 20-fold, 50-fold, or 100-fold in α-MEM-20% FCS. Aliquots (0.1 ml) of these suspensions were plated in microtiter wells and allowed to incubate for 1 hr at 37°C in a humid 4% CO₂-air atmosphere. The remaining marrow cells were placed in 35-mm tissue culture plates for adherent cell depletion, as described above.

The nonadherent cells were collected from the 35-mm culture plates and the plates washed 3 times with α-MEM-20% FCS. The nonadherent cells were then washed twice with α-MEM and counted. The number of adherent cells and the percentage of adherent cells in the marrow mononuclear cell preparation were determined. The 35-mm tissue culture plates containing the marrow adherent cells were stained in situ with Wright’s Giemsa stain, and a 200-cell count differential was performed by light microscopy. The 35-mm tissue culture plates were also stained with nonspecific esterase to determine the number of monocyte-macrophages present in the adherent cell population.

The microtiter wells, which contained varying dilutions of the unfractionated marrow, were washed 3 times with α-MEM–20% FCS, and the washes discarded. The adherent cells in the microtiter plates were covered with α-MEM–20% FCS until used.

Assay of CFU-E and BFU-E

Unfractionated marrow that had been kept on ice, or nonadherent marrow, was cultured in plasma clots as described by Tepperman et al. Forty to sixty thousand cells were cultured in 0.1 ml plasma clots with 1 IU/ml sheep (Connaught) or human urinary erythropoietin (generously provided by the NIHHLB). In coculture studies of marrow-adherent cells and nonadherent marrow, 4–6 × 10^6 cells were suspended in plasma clot media as described above, and the clots formed in the microtiter wells that contained varying concentrations of adherent cells. Plasma clots formed within 30 sec of plating. The plates were incubated in a humid atmosphere of 4% CO₂-air at 37°C for 7 days for CFU-E or 14 days for BFU-E. All assays were done in quadruplicate. No erythroid colony or burst formation occurred when adherent cells were cultured alone. At the end of the culture period, clots were applied to glass slides, fixed, and stained with benzidine and hematoxylin. CFU-E- and BFU-E-derived colonies and bursts were counted microscopically.

Therefore, in each experiment, erythroid colony and burst formation were determined in cultures of unfractionated marrow, nonadherent marrow, and nonadherent marrow cocultured with marrow-adherent cells similar to the numbers of adherent cells present in the unfractionated marrow or diluted up to 1/100 of the number of adherent cells present in the unfractionated marrow. In selected experiments, nonadherent marrow was cocultured with allogeneic adherent cells. Results were calculated as total erythroid colonies or bursts per assay and per 10^6 unfractionated marrow cells or nonadherent marrow cells plated. Results were normalized by dividing results of individual patient cultures by the number of colonies or bursts formed by the patient’s unfractionated marrow and multiplying by 100. This was done because of the large variations in colony formation seen in cultures from different patients (18–700 colonies/10^6 cells plated).

Experiments With Conditioned Media

Conditioned medium was prepared by incubating 4–6 × 10^3/ml unfractionated marrow mononuclear cells, nonadherent marrow, or marrow-adherent cells in α-MEM–20% FCS for 1 hr to 7 days at 37°C in a humid atmosphere of 4% CO₂-air. Cells were removed by centrifugation, and the media recovered. One-tenth milliliter of conditioned medium per milliliter was added to cultures of unfractionated marrow or nonadherent marrow as described above.

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### Statistical Methods

Data were compared using a two-way analysis of variance and covariance for repeated measures as well as paired t tests. Differences were only considered significant for \( p < 0.05 \). Normalization of the erythroid colony or burst data did not affect the results and showed similar patterns of statistical significance.

### RESULTS

The percentages of adherent cells present in marrow samples from patients with anemia of chronic disease, control patients, and normals \((38 \pm 8\) for anemic patients, \(44 \pm 8\) for control patients, and \(40 \pm 5\) for normals) did not differ significantly \((p = 0.8)\). The marrow-adherent cell populations from anemic patients, control patients, and normals were also similar in morphology and relative numbers. The adherent cells were predominantly monocyte-macrophages \((89\% \pm 2\% \) for anemic patients versus \(79\% \pm 6\% \) for control patients and normals) and did not differ significantly \((p = 0.2)\).

The effects of adherent cell depletion of marrow mononuclear cells and coculture of \(6 \times 10^4\) nonadherent marrow cells with autologous adherent cells on erythroid colony formation in normal, control patient, and anemic patient marrow cultures are shown in Fig. 1. The number of erythroid colonies formed in cultures of unfractionated marrow from patients with the anemia of chronic disease was extremely variable. Erythroid colony formation from unfractionated marrow ranged from 18 to 692 colonies/\(10^5\) cells plated, with a median value of 212 erythroid colonies/\(10^5\) cells plated. Five of the ten anemic patients formed fewer than 150 colonies/\(10^5\) cells plated. Removal of marrow-adherent cells increased erythroid colony formation in 8 of the 10 patients and did not affect erythroid formation in one patient (patient 1). In one patient (patient 7), removal of marrow-adherent cells decreased erythroid colony formation, but this decrease was not significant \((p > 0.05)\). The median value for erythroid colony formation in nonadherent anemic marrow cultures was 338/\(10^5\) cells plated. Coculture of autologous marrow-adherent cells with nonadherent marrow cells from patients with the anemia of chronic disease significantly decreased erythroid colony formation in 9 of the 10 patients studied. In one patient (patient 7), coculture of marrow-adherent cells with autologous nonadherent marrow cells increased erythroid colony formation, but this increase was not significant \((p > 0.05)\). All anemic patient culture studies were then compared after nor-

### Figure 1

![Erythroid colony formation in cultures of unfractionated marrow, nonadherent marrow, and nonadherent marrow cocultured with autologous adherent cells from normals, control patients, and patients with anemia of chronic disease. Unfractionated marrow (M), nonadherent marrow (N), and marrow-adherent cells (A) were cultured as described in Materials and Methods. Results are presented as the average erythroid colony formation per \(10^5\) cells plated of quadruplicate samples of unfractionated marrow, nonadherent marrow, and nonadherent marrow cells cocultured with autologous marrow-adherent cells for each subject studied. The numbers depicting control patients and anemic patients correspond to the patient numbers given in Table 1. The numbers given for normals represent the 5 normal volunteers studied. The bars show the median value for erythroid colony formation in cultures of unfractionated marrow, nonadherent marrow, and nonadherent marrow cocultured with autologous adherent cells for normals, control patients, and patients with the anemia of chronic disease.](image-url)
malizing each patient's culture results by dividing the number of colonies formed per $10^5$ cells in the patient's nonadherent marrow culture and cultures containing nonadherent marrow cocultured with marrow adherent cells, by the number of colonies formed in that patient's unfractinated marrow. As seen in Fig. 2, adherent cell depletion of anemic patient marrow significantly increased (54% ± 23%) erythroid colony formation ($p = 0.04$). Coculture of autologous marrow-adherent cells with nonadherent anemic marrow cells significantly decreased (62% ± 12%) erythroid colony formation ($p = 0.01$). This inhibition of erythroid colony formation by autologous marrow-adherent cells from anemic patients decreased with decreasing concentrations of adherent cells present in the cultures (Fig. 3). For example, cultures containing one-fifth the concentration of adherent cells as present in the unfractinated marrows (A/5), decreased the suppression of erythroid colony formation from 54% to 29%. Coculture of autologous marrow-adherent cells with nonadherent anemic marrow cells decreased erythroid colony formation regardless of the erythropoietin concentration present in the cultures (50–3,000 mU/ml, data not shown).

While adherent cells suppressed erythroid colony formation in anemic patients, removal of marrow-adherent cells and coculture of these cells with autologous nonadherent marrow cells from control patients had a different effect (Fig. 1). In two control patients (patients 11 and 14), adherent cell depletion modestly decreased erythroid colony formation; in two control patients (patients 15 and 17), adherent cell depletion did not affect erythroid colony formation; and in three control patients (patients 12, 13, and 16), adherent cell depletion modestly increased erythroid colony formation. These changes in erythroid colony formation were not statistically significant. When data for all control patients were compared (Fig. 2), adherent cell depletion of control patient marrow did not significantly affect erythroid colony formation ($p = 0.33$). Coculture of autologous adherent cells with control patient nonadherent marrow also had a variable and insignificant effect on erythroid colony formation ($p = 0.23$), increasing it 10% ± 8% (Fig. 2). The median values for erythroid colony formation per $10^5$ cells for control patient unfractinated marrow (296), nonadherent marrow (304), and nonadherent marrow cocultured with autologous adherent cells (293) were similar.

In contrast, adherent cell depletion of normal marrow showed another pattern of response (Fig. 1). Adherent cell depletion decreased erythroid colony formation in all five normal marrows tested. Adherent cell depletion significantly decreased (37% ± 8%) normal erythroid colony formation ($p = 0.01$) when all normals were compared (Fig. 2). Coculture of autologous adherent cells with normal volunteer nonadherent marrow consistently increased erythroid colony formation in all 5 normals (Fig. 1), and significantly increased (47% ± 12%) erythroid colony formation ($p = 0.02$) when all normals were compared (Fig. 2). Erythroid burst formation was not significantly or consistently affected by adherent cell depletion or addition for any of the groups tested (data not shown).

An analysis of covariance for repeated measures demonstrated that the effects of adherent cells from patients with the anemia of chronic disease on erythroid colony formation was markedly different from those seen in control patients or normal volunteers ($p < 0.0001$). Adherent cells suppressed erythroid col-

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![Fig. 2. Erythroid colony formation in cultures of unfractionated marrow, nonadherent marrow, and nonadherent marrow cocultured with autologous adherent cells from normals, control patients, and patients with anemia of chronic disease. Unfractionated marrow (M), nonadherent marrow (N), and marrow-adherent cells (A) were cultured as described in text. Results for each patient were normalized by dividing the number of erythroid colonies formed in nonadherent marrow cultures, with or without adherent cells, by the number of erythroid colonies formed in cultures of unfractionated marrow. These results were then compared and are reported as the mean ± SEM. (*) Significantly different from unfractionated marrow cultures. (**) Significantly different from nonadherent marrow cultures.](image)
Fig. 3. Effects of decreasing the concentration of autologous marrow-adherent cells cocultured with nonadherent marrow cells from patients with the anemia of chronic disease. Nonadherent marrow (N) was cultured with or without varying concentrations of marrow adherent cells (A) as described in Materials and Methods. Results were normalized as described in the legend to Fig. 2. Results are reported as mean ± SEM for six patients. (*) Significantly different from nonadherent marrow cultures.

Table 2. Erythroid Colony Formation in Nonadherent Marrow Cocultured With Autologous or Allogeneic Marrow-Adherent Cells

<table>
<thead>
<tr>
<th>Source of Nonadherent Marrow</th>
<th>Control Patients</th>
<th>Anemic Patients</th>
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<tr>
<td>Nonadherent marrow</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Nonadherent marrow and</td>
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<td>control patient adherent cells</td>
<td>146 ± 22</td>
<td>117 ± 31</td>
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<tr>
<td>Nonadherent marrow and</td>
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<tr>
<td>anemic patient adherent cells</td>
<td>116 ± 7</td>
<td>49 ± 7*</td>
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Results from each experiment were normalized by dividing the number of colonies formed per 10^5 cells in each coculture experiment by the number of colonies formed when nonadherent marrow cells from the patient were cultured alone. Results are presented as the mean ± SEM for three independent experiments done in quadruplicate.

*p < 0.05 compared to results from nonadherent marrow cultures.

**DISCUSSION**

Marrow-adherent cells are predominantly (84% in our study) monocyte-macrophages. Chronic inflammation, tumor cells, and a variety of antigenic stimuli activate macrophages to produce substances that can affect cell proliferation and other cellular processes. We postulated that macrophages from patients with anemia of chronic disease should be "activated" by the chronic inflammatory or malignant process and would inhibit erythroid progenitor cell proliferation and differentiation. Other workers had previously demonstrated that interferons and lipoproteins produced by activated macrophages, suppress erythroid colony formation.

Our results demonstrate that under the conditions we employed, marrow-adherent cells from patients with the anemia of chronic disease significantly inhibit erythroid colony formation (p = 0.04) in vitro. The effect of anemia of chronic disease adherent cells on autologous erythroid colony formation is markedly different than that seen with coculture of adherent cells from control patients or normal volunteers with autologous marrow (p = 0.0004). This difference is significant, even when corrected for variations in overall marrow erythroid colony formation among anemia of chronic disease patients, control patients, and normals (p < 0.0001). Adherent cell depletion of control patient marrow did not significantly increase erythroid colony formation (p = 0.33). In contrast, adherent cell depletion of normal marrow significantly decreased erythroid colony formation (p < 0.01). The number of CFU-E/10^5 cells plated should be increased when adherent cells are removed from unfractonated marrow, because adherent cells do not form colonies and constitute approximately 30%-40% of marrow mononuclear cells. One would expect approximately a 1.5-fold increase in erythroid colony formation in cultures using nonadherent marrow cells when similar numbers of nonadherent and unfractonated marrow cells are cultured. These data suggest that marrow-adherent cells are normally required for optimal erythroid col-
Further support for the stimulatory effect of adherent cells on normal erythroid colony formation was seen when normal adherent cells were cocultured with autologous nonadherent marrow. Under these circumstances, erythroid colony formation was significantly enhanced ($p = 0.01$). Interestingly, addition of control patient adherent cells to autologous nonadherent marrow cultures had a variable effect on erythroid colony formation and, overall, did not stimulate erythroid colony formation ($p = 0.23$). These results demonstrate that marrow samples from control patients with cancer who are "hematologically normal" are different from normal marrow and raise questions as to their suitability as "normal marrow" donors for experiments.

We propose that suppression of CFU-E by marrow-adherent cells in patients with anemia of chronic disease results from activation of the macrophage by the underlying disease process. A soluble inhibitor appears to be involved in the in vitro suppression of CFU-E from patients with the anemia of chronic disease, because our plasma clots were cultured over a feeder layer of adherent cells. It is unlikely that the marrow CFU-E suspended in a semisolid clot make direct cell-to-cell contact with the marrow macrophages adherent to the bottom of the microtiter wells. Conditioned media from marrow, nonadherent marrow, or adherent cells from patients with anemia of chronic disease did not consistently suppress CFU-E in adherent-cell-depleted marrow cultures from patients with anemia of chronic disease or normals. These results suggest that the inhibitory substance may have an extremely short half-life in vitro because testing media conditioned for 2 hr or up to 7 days by adherent cells from patients with the anemia of chronic disease did not result in suppression of CFU-E. Alternatively, the inhibitor may be patient-specific. The inhibitor appears to be specific for the patient's CFU-E, since adherent cells from patients with the anemia of chronic disease only suppressed autologous marrow CFU-E. Host-specific effects of macrophages have been reported in other systems. For example, macrophage activation of T lymphocytes has been shown to be host-specific.

Marrow-adherent cells from control patients with malignancies did not significantly suppress or stimulate autologous CFU-E, in contrast to results seen with normal or anemia of chronic disease marrow-adherent cells. This lack of stimulation or suppression of CFU-E by marrow-adherent cells from control patients with chronic diseases may simply reflect a difference in the degree of macrophage activation in these patients or differences in the percent of macrophages activated compared to patients with the anemia of chronic disease.

Zanjani et al. have recently reported adherent cell suppression of marrow CFU-E in some patients with disseminated fungal infections. In contrast to our results, they demonstrated that adherent cells from some of these patients could suppress normal marrow erythroid colony formation. They also reported that adherent cell conditioned media could mimic the effects of adherent cells. None of the patients we studied had disseminated fungal infections. These data suggest the adherent cells from patients with disseminated fungal infections may produce a less specific erythroid inhibitor than that seen in patients with other chronic diseases.

ACKNOWLEDGMENT

We would like to thank Judi Skinner for her preparation of the manuscript.

REFERENCES

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