Human Bone Marrow Depleted of CD33-Positive Cells Mediates Delayed but Durable Reconstitution of Hematopoiesis: Clinical Trial of MY9 Monoclonal Antibody-Purged Autografts for the Treatment of Acute Myeloid Leukemia

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The CD33 antigen, identified by murine monoclonal antibody anti-MY9, is expressed by clonogenic leukemic cells from almost all patients with acute myeloid leukemia; it is also expressed by normal myeloid progenitor cells. Twelve consecutive patients with de novo acute myeloid leukemia received myeloablative therapy followed by infusion of autologous marrow previously treated in vitro with anti-MY9 and complement. Anti-MY9 and complement treatment eliminated virtually all committed myeloid progenitors (colony-forming unit granulocyte-macrophage) from the autografts. Nevertheless, in the absence of early relapse of leukemia, all patients showed durable trilineage engraftment. The median interval post bone marrow transplantation (BMT) required to achieve an absolute neutrophil count greater than 500/μL was 43 days (range, 18 to 75), to achieve a platelet count greater than 20,000/μL without transfusion was 92 days (range, 35 to 679), and to achieve red blood cell transfusion independence was 105 days (range, 37 to 670). At the time of BM harvest, 10 patients were in second remission, one patient was in first remission, and one patient was in third remission.

Eight patients relapsed 3 to 18 months after BMT. Four patients transplanted in second remission remain disease-free 34+, 37+, 52+, and 57+ months after BMT. There was no treatment-related mortality. Early engraftment was significantly delayed in patients receiving CD33-purged autografts compared with concurrently treated patients receiving CD9/CD10-purged autografts for acute lymphoblastic leukemia or patients receiving CD6-purged allografts from HLA-compatible sibling donors. In contrast, both groups of autograft patients required a significantly longer time to achieve neutrophil counts greater than 500/μL and greater than 1,000/μL than did patients receiving normal allogeneic marrow. CD33+ committed myeloid progenitor cells thus appear to play an important role in the early phase of hematopoietic reconstitution after BMT. However, our results also show that human marrow depleted of CD33+ cells can sustain durable engraftment after myeloablative therapy, and provide further evidence that the CD33 antigen is absent from the human pluripotent hematopoietic stem cell.

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marrow of myeloid leukemia cells. However, the CD33 antigen is also present on normal early myeloid progenitors, including more than 90% of colony-forming unit granulocyte-macrophage (CFU-GM), ~50% of burst-forming unit erythroid (BFU-E), and ~50% of CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM).23,27,28 None-theless, human marrow depleted of CD33+ cells in vitro is able to grow normally in long-term BM cultures.8,20 Furthermore, the earliest identifiable hematopoietic progenitor cells appear not to express the CD33 antigen,29-31 although they do express CD34 (MY10).29-32 These data suggest that CD33 MoAb-purged marrow should be able to sustain adequate hematopoiesis after myeloablative chemotherapy. We describe hematopoietic reconstitution of 12 patients with AML who received high-dose ablative therapy followed by reinfusion of autologous BM treated in vitro with CD33 MoAb and complement.

PATIENTS AND METHODS

Patient selection. Patients were considered eligible for this study if they were less than 50 years of age, did not have an appropriate HLA-compatible sibling donor for allogeneic BMT, and showed no major impairment of cardiac, pulmonary, renal, or hepatic function. The diagnosis of AML was confirmed in all patients by examination of BM aspirates or biopsies, and all patients had leukemic blasts that expressed the CD33 antigen as determined by flow cytometry. The treatment protocol was approved by the Institutional Review Board of the Dana-Farber Cancer Institute and informed consent was obtained from all patients.

Anti-MY9 MoAb and complement. The murine CD33 MoAb anti-MY9 has been described in detail elsewhere.23 Antibody was isolated from ascites fluid produced by mice bearing hybridoma tumors, filtered, frozen, and stored at −70°C until used for marrow treatment. Batches of rabbit complement (Pel-Freeze, Brown Deer, WI) were tested for optimal specific cytotoxicity.33

Collection and processing of BM. CR was confirmed before harvesting of the marrow autografts. BM was obtained by multiple percutaneous aspirations from the posterior iliac crests of patients under general anesthesia.34 Marrow was collected in sterile RPMI-1640 medium with preservative-free heparin, passed through graded stainless steel mesh filters, washed, and concentrated into a buffy coat using the COBE 2991 cell washer (COBE Laboratories, Lakewood, CO). BM mononuclear cells were then isolated by Ficoll-Hypaque density gradient centrifugation, washed, and resuspended at a concentration of 2 × 10^7 cells/mL. These cells were subjected to three sequential treatments, each consisting of 15 minutes of incubation with anti-MY9 MoAb at 4°C followed by incubation with baby rabbit complement at 37°C for 30 minutes. After treatment, the BM cells were cryopreserved in the vapor phase of liquid nitrogen at −196°C in medium containing 10% dimethyl sulfoxide and 90% autologous serum. Sterility of the autografts after purging was routinely confirmed by performing cultures for bacteria and fungi.

Preparative regimens and marrow reinfusion. The preparative regimen originally included high-dose cytarabine because of its demonstrated efficacy in the treatment of AML.4 For patients received cytarabine 3,000 mg/m² body surface area intravenously (IV) every 12 hours for 8 doses (24 g/m² total) over 4 consecutive days) followed by total body irradiation (TBI). Due to significant toxicity from previous high-dose cytarabine therapy, four patients received instead cyclophosphamide 60 mg/kg body weight IV daily for 2 consecutive days (120 mg/kg total) followed by TBI. Since June 1988, all patients have received busulfan 1 mg/kg orally every 6 hours for 16 doses (16 mg/kg total over 4 consecutive days) followed by cyclophosphamide on 2 consecutive days as described above. Continuous bladder irrigation through an indwelling catheter was undertaken in all patients receiving cyclophosphamide to reduce the risk of hemorrhagic cystitis; it was discontinued 12 hours after the last dose of cyclophosphamide. TBI (10 cGy/min) was administered twice daily in equal fractions over 3 to 4 days (total of six to eight fractions). Three patients received a total of 1,200 cGy in 200-cGy fractions, whereas five received 1,400 cGy in 175-cGy fractions. For all patients, cryopreserved autologous marrow that had been previously treated in vitro with MoAb and complement was thawed rapidly and reinfused through a central venous catheter on day 0. Patients receiving TBI underwent marrow reinfusion within 24 hours of the final fraction of TBI, while patients receiving the busulfan/cyclophosphamide regimen underwent marrow reinfusion 2 days after the last dose of chemotherapy.

Supportive care. Indwelling central venous catheters were placed in all patients before transplantation. Patients received posttransplant care in single rooms under conditions of strict reverse isolation; sterile environments were not used. Prophylactic oral ciprofloxacin or trimethoprim-sulfamethoxazole was prescribed for all patients, but was discontinued when patients required parenteral antibiotics. All but the first two patients also received prophylactic acyclovir (5 mg/kg IV or 400 mg orally every 8 hours). Empiric broad-spectrum antibacterial therapy was instituted promptly when granulocytopenic patients became febrile. Empiric antifungal therapy was generally instituted if patients remained febrile despite receiving antibacterial therapy for 4 days. All patients received single donor platelet transfusions from cytomegalovirus seronegative donors to maintain a platelet count of at least 20,000/µL and frozen deglycerolized red blood cells (RBCs) to maintain a hematocrit of at least 28%. All blood products were irradiated to avoid transfusion-related alloreactive disease.35 Reverse isolation and empiric antibiotic therapy were usually maintained until patients were afebrile and demonstrated a sustained absolute neutrophil count in excess of 500/µL. After hospital discharge, oral trimethoprim-sulfamethoxazole was prescribed until 12 months posttransplant to prevent Pneumocystis carinii infection.

Criteria for engraftment. Engraftment was assessed by determining the number of days after marrow reinfusion required to achieve different peripheral blood cell counts. Neutrophil engraftment was said to have occurred on the first of 2 consecutive days that the total neutrophil count exceeded 100/µL, 500/µL, or 1,000/µL. Platelet engraftment was said to have occurred on the first day that the platelet count exceeded 20,000/µL or 40,000/µL for 2 weeks in the absence of platelet transfusions. Erythrocyte engraftment was scored on the first day that the hematocrit exceeded 30% for 2 weeks in the absence of RBC transfusions.

Hematopoietic progenitor assays. Aliquots of mononuclear cells obtained from the BM grafts before and after in vitro treatment with anti-MY9 and complement were plated in agar for CFU-GM as previously described.23 Conditioned medium (20%) from the bladder carcinoma cell line 5637 was added as a source of colony-stimulating factor. Assays were performed in quadruplicate in 24-well plastic culture plates (Linbro; Flow Laboratories, McLean, VA) and incubated for 14 days in 5% CO₂ humidified air at 37°C. Colonies of granulocytes and/or macrophages containing greater than 40 cells were scored as CFU-GM. The total CFU-GM content of marrow autografts before and after MY9 purging was calculated by multiplying the number of colonies scored per number of cells cultured by the number of marrow mononuclear.
CD33 MoAb PURGED AUTOGRRAFTS FOR AML

Differences in the time to neutrophil engraftment of patients receiving CD33-treated autografts, CD9/CD10-treated autografts, and CD6-treated allografts were calculated by the Wilcoxon test and the log-rank test. All P values reported are from the Wilcoxon analysis, because it had more statistical power to discriminate early differences between the relatively small groups of autograft patients. Means and standard deviations (SD) were calculated on a Macintosh SE computer (Apple Computer, Cupertino, CA) using the StatView software program (Brainpower Inc, Calabasas, CA). Results were analyzed as of November 1991.

RESULTS

Patient characteristics. Twelve consecutive patients with de novo AML received anti-MY9-treated autografts between December 1985 and June 1989 (Table 1). The median age was 40 years (range, 25 to 49). Central nervous system (CNS) involvement with leukemia had not been documented in any patient. Two patients (unique patient number [UPN] 1082 and 1242) had shown granulocytic sarcomas at some point during their clinical course, involving nasal and subcutaneous sites, respectively. All 12 patients had received extensive prior therapy, receiving a median of 8 cycles (range 4 to 15) of intensive chemotherapy for remission induction and postremission treatment. All but three patients had received regimens containing high-dose cytarabine. The median duration of first remission in relapsed patients was 14 months (range, 4 to 42 months). The median interval between attaining pretransplant remission and undergoing marrow harvest was 2 months (range, 1 to 6 months). At the time of marrow harvest, 10 patients were in second remission, one patient was in third remission, and one patient was in first remission. The interval between marrow harvest and marrow reinfusion was less than 4 weeks in all patients except one (UPN 1310) who relapsed shortly after marrow harvest. After successful induction of a third remission, this patient underwent transplantation 14 weeks after marrow harvest.

Autograft characteristics. The median number of total nucleated BM cells harvested was $2.4 \times 10^{10}$ (range, $1.5 \times 10^{10}$ to $5.1 \times 10^{10}$ ) and the median number of mononuclear cells treated with anti-MY9 and complement was $6.8 \times 10^{9}$ (range, $2.7 \times 10^{9}$ to $28.0 \times 10^{9}$). The median number of mononuclear cells reinfused after marrow thawing was $2.7 \times 10^{9}$ (range, $0.6 \times 10^{9}$ to $12.4 \times 10^{9}$), representing $4.0 \times 10^{7}$/kg body weight (range, $1.0 \times 10^{7}$ to $14.6 \times 10^{7}$/kg). It should be noted that the number of cells infused in these MY9-purged autografts did not differ markedly from that infused in allografts from healthy sibling donors at our institution (median, $4.5 \times 10^{7}$ cells/kg). The CFU-GM content of harvested marrow was variable among patients (Table 2). BM mononuclear cells plated before purging generated $38.8 \pm 16.5$ (mean $\pm$ SD) day 14 CFU-GM per $5 \times 10^{4}$ cells, indicating autograft progenitor contents of $868 \pm 793 \times 10^{2}$ CFU-GM/kg patient body weight. After purging, only $0.2 \pm 0.4$ colonies per $5 \times 10^{4}$ cells ($2.2 \pm 3.8 \times 10^{2}$ total autograft CFU-GM/kg) remained (Table 2). Thus, anti-MY9 and complement treatment eliminated $99.7\% \pm 0.6\%$ of day 14 CFU-GM from the autografts. Before marrow treatment, $32\% \pm 20\%$ of marrow mononuclear cells expressed the CD33 antigen by indirect immuno-fluorescence analysis. This does not differ substantially from the expected expression of CD33 by normal BM cells. Residual CD33 cells could not be reliably quantified by flow cytometry after marrow treatment because the complement lysis procedure induces high background staining. Depletion of CFU-GM was therefore used to assess indirectly the efficiency of the in vitro purging technique.

Hematopoietic reconstitution. All patients demonstrated durable engraftment of granulocytes; the median time to achieve an absolute neutrophil count (ANC) in excess of 500/µL was 43 days after marrow infusion (range, 16 to 75; Fig 1B). Eleven patients attained platelet transfusion independence. The median time after BMT to achieve platelet counts greater than 20,000/µL and 40,000/µL were 92 days (range, 35 to 679) and 120 days (range, 49 to 695), respectively. Nine patients achieved a stable hematocrit greater than 30% in the absence of RBC transfusions at a

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Abbreviations: FAB, French-American-British Working Group classification.
Table 2. Effect of Marrow Treatment on CFU-GM

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<td>Mean ± SD</td>
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<td>868 ± 793</td>
<td>0.2 ± 0.4</td>
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Abbreviation: ND, not done.
*Number of colonies scored per 5 x 10⁴ marrow mononuclear cells plated.
†Number of CFU-GM/kg x 10⁻² (calculated as described in Patients and Methods).

To assess the contribution of CD33⁺ marrow cells to hematopoietic reconstitution after BMT, we compared neutrophil engraftment in three groups of patients who underwent transplantation concurrently at the Dana-Farber Cancer Institute: the 12 patients who received CD33-purged autografts, 18 adults with acute lymphoblastic leukemia (ALL) who received CD9/CD10-purged autografts, and 43 adults with AML or ALL who received allografts from HLA-compatible sibling donors. Allografts were depleted of CD6⁺ T cells to prevent graft-versus-host disease; prophylactic immunosuppressive drugs were not prescribed after BMT. All allografts and autografts were treated in vitro with MoAb and complement using a standardized protocol. Furthermore, patients in all three groups received uniform supportive care after BMT, and none were treated with recombinant hematopoietic growth factors to accelerate engraftment. As expected, neutrophil
engraftment was delayed in patients receiving marrow grafts treated with CD33 MoAb and complement compared with patients receiving grafts treated with MoAb that do not react with normal myeloid progenitors (Fig 1). The median interval post-BMT required to achieve an ANC of 100/μL was 29 days for CD33-depleted autograft patients, compared with 16 days for the ALL autograft patients and 15 days for the allograft patients (Fig 1A). Furthermore, the AML autograft patients required a median of 43 days to achieve neutrophil counts in excess of 500/μL, whereas the ALL autograft patients required 29 days and the allograft patients only 21 days (Fig 1B). The interval required to achieve neutrophil counts ≥1,000/μL was 63 days for the AML autograft patient, 50 days for the ALL autograft patients, and 32 days for the allograft patients (Fig 1C). Each of the differences between CD33-purged autograft patients and allograft patients was highly statistically significant by the Wilcoxon test (P = .0001). When comparing AML autograft patients and ALL autograft patients, however, the only significant difference (P = .008) was in the time to achieve an ANC greater than 100/μL; the numbers of days required to achieve an ANC greater than 500/μL or greater than 1,000/μL were statistically indistinguishable (P = .4 for both).

Posttransplant clinical course and toxicity. The BM harvest procedure was well-tolerated by all patients and there were no significant complications of marrow infusion. All patients became febrile while granulocytopenic; the median duration of parenteral antibiotic therapy was 36 days (range, 15 to 64). Eight patients also received empiric amphotericin B therapy for fevers unresponsive to antibacterial therapy. Nine episodes of bacteremia occurred in eight patients, including Staphylococcus epidermidis (four episodes), Klebsiella (two episodes), and one episode each of Escherichia coli, Stomatococcus mucilaginosus, and group A β hemolytic Streptococcus. These infections resolved with appropriate antibiotic therapy, although central line removal was required in one patient. Invasive fungal disease (disseminated Fusarium infection) was documented in only one patient; this infection was eventually eradicated by a combination of prolonged amphotericin therapy and surgery.42 One patient experienced severe bleeding from multiple gastric ulcers that required surgical intervention. There were no episodes of acute cardiomyopathy, hemorrhagic cystitis, busulfan-associated seizure, veno-occlusive disease of the liver, interstitial pneumonitis, or posttransplant hematolytic-uremic syndrome. Despite the prolonged pancytopenia experienced by these patients, there were no transplant-related deaths. The median duration of hospitalization for BMT was 47 days; patients were generally discharged within 5 days of granulocyte recovery.

Leukemic relapses and DFS. Eight patients have relapsed at a median of eight months (range, 3 to 18) after BMT, including the one patient transplanted in first remission and the two patients transplanted in third remission. Three patients who relapsed post-BMT had received the cytarabine/TBI preparative regimen, three had received cyclophosphamide/TBI, and two had received busulfan/cyclophosphamide. In all cases studied, leukemic blasts isolated at posttransplant relapse were morphologically and immunophenotypically similar to those obtained at diagnosis or pre-BMT relapse. In particular, myeloblasts from patients in relapse post-BMT expressed the CD33 antigen, making it unlikely that CD33 MoAb purging resulted in selection for a CD33− subset of leukemic progenitor cells. No extramedullary involvement was evident at the time of hematologic relapse in these patients. Survival after relapse was generally brief (median, 3 months) whether or not further antileukemic therapy was undertaken. Only one patient could be successfully induced into another CR with standard chemotherapy. Four patients transplanted in second remission remain alive and disease-free at 34+, 37+, 52+, and 57+ months post-BMT. For each of these patients, the duration of remission since BMT exceeds the duration of their first remission. Actuarial analysis indicates a DFS survival of 33% ± 14% (mean ± SE) at greater than 3 years posttransplant (Fig 2).

**DISCUSSION**

The CD33 antigen is a 67-Kd cell surface glycoprotein encoded by an approximately 35-kb gene located on the long arm of human chromosome 19q41; its function on myeloid cells is not known. The CD33 antigen is expressed by normal committed progenitor cells, including CFU-GEMM, CFU-GM, and BFU-E.23,28,30 Nevertheless, we found that marrow autografts depleted of CD33+ cells could mediate durable reconstitution of hematopoiesis after myeloablative therapy. These in vivo results are in good agreement with previous in vitro studies, which demonstrated normal growth of CD33− cells in long-term cultures.29,32 Our results thus provide clinical evidence that the CD33 antigen is absent from the human pluripotent hematopoietic stem cell. However, hematopoietic reconstitution after infusion of CD33-purged autografts was rather prolonged. As summarized in Table 3, neutrophil and platelet engraftment of our patients appeared to be delayed relative to that of AML patients receiving unpurged autografts,10,44 CD14/CD15 MoAb-purged autografts,9,26 or autografts purged with cyclophos-
Comparison of hematologic engraftment in our patients to that of AML patients receiving antimyeloid MoAb and complement. Previous allograft patients were treated with CD14 and CD15 MoAb and complement. Previous in vitro studies suggest that CD33 purging should deplete more normal committed myeloid progenitors than CD14 and CD15 purging. Indeed, treatment with CD14/CD15 MoAb and complement eliminated only 0% to 83% (median, 55%) of CFU-GM from autografts, whereas treatment with CD33 MoAb and complement eliminated 99.7% ± 0.6% of CFU-GM. Both the Dartmouth group and the Hopkins group have reported a significant correlation between the rapidity of engraftment and the CFU-GM content of purged autografts. The virtual elimination of CFU-GM from CD33-depleted autografts may thus explain the delayed engraftment in our patients compared with that seen in other series.

To define further the role of CD33+ cells in hematopoietic reconstitution after BMT, we compared neutrophil engraftment in CD33 autograft patients with that of concurrently treated patients at our institution who received marrow grafts treated with MoAb that do not recognize myeloid antigens. In contrast to treatment with CD33 MoAb, marrow treatment with either CD6 MoAb or CD9 and CD10 MoAb and complement does not deplete normal myeloid progenitors (our unpublished results). Like the autograft patients, the allograft patients had received intensive chemotherapy for the treatment of their leukemia before BMT; in contrast to the former, however, they received normal allogeneic marrow that had not been exposed to myelotoxic drugs. Furthermore, our CD6-depleted allograft patients are not prescribed immunosuppressive or myelosuppressive agents routinely after BMT. Patients receiving CD33-purged autografts achieved a neutrophil count of 100/mL approximately 2 weeks later than either our allograft patients or ALL autograft patients (Fig 1A). Although early engraftment in ALL autograft patients was similar to that of patients receiving normal allogeneic marrow, ALL autograft patients took longer than the allograft patients to achieve neutrophil counts in excess of 500/mL and 1,000/mL. Indeed, later neutrophil engraftment of patients receiving CD9/CD10-purged autografts was not significantly different from that of patients receiving CD33-purged autografts (Fig 1B and C). Depletion of CD33+ myeloid progenitors from marrow grafts is therefore associated with a selective delay in the early phases of neutrophil engraftment; we have no evidence to suggest that later phases of engraftment depend on cells expressing CD33. The delay in later engraftment of both AML and ALL autograft patients may be due to quantitative or qualitative defects in more primitive, CD33- hematopoietic precursor cells; such defects could result from previous cytotoxic therapy or the leukemia itself. Taken together, our results support the hypothesis that CD33+-committed myeloid progenitor cells, although crucial in the early phases of engraftment after BMT, are not required for eventual recovery of hematopoiesis. Experiments in mice have also shown that engraftment after BMT occurs in two phases: an early, transient phase due to committed progenitor cells, and a more delayed durable phase resulting from true pluripotent stem cells.

Instead of eliminating malignant cells from the marrow autografts of patients with cancer, some investigators have infused positively selected autologous hematopoietic precursor cells after myeloablative therapy. Such marrow cells are likely to comprise both CD34+ CD33+ committed myeloid progenitor cells and more immature CD34+ CD33- hematopoietic precursors. Based on our results, one would expect patients receiving predominantly CD34+ CD33- cells to experience delayed engraftment due to the paucity of committed myeloid progenitors infused. Indeed, relatively slow hematopoietic reconstitution was observed in some of the patients receiving purified CD34+ marrow cells. These results have obvious implications for clinical studies using positively selected precursor cells to reconstitute hematopoiesis.

Although our patients experienced prolonged pancytopenia after high-dose ablative therapy, there were no infectious deaths on this study. Only one patient developed...
invasive fungal disease, and this infection was eradicated by aggressive treatment. Clinically significant bleeding occurred in only one patient. Other potentially lethal complications of BMT were not seen; all deaths resulted from relapsed leukemia. The absence of infectious deaths is perhaps remarkable, in view of the relatively prolonged time to engraftment. However, the ANC of these patients ranged from 100 to 499/μL for ~2 weeks before stable counts in excess of 500/μL were achieved (Fig 1). Even small numbers of recovering granulocytes might contribute to host defenses, thus accounting for the relative rarity of major infectious complications in these patients.

This phase I clinical trial also showed that high-dose ablative therapy with reinfusion of CD33 MoAb-purged marrow can be an effective treatment for advanced AML. Four patients treated in second remission remain disease-free ~3 to 5 years post-BMT, and in each case duration of remission since BMT has exceeded that of first remission. The actuarial DFS of 33% ± 14% at greater than 3 years compares favorably with autologous6,7 and allogeneic8-15 transplant series involving similar patients. Because a relatively small number of patients have been treated and heterogeneous preparative regimens were used, however, definitive conclusions regarding the antileukemic efficacy of this treatment approach cannot be made. Further patient accrual and longer follow-up should permit a more conclusive assessment of the therapeutic efficacy of anti-MY9-purged autografts for AML.

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Human bone marrow depleted of CD33-positive cells mediates delayed but durable reconstitution of hematopoiesis: clinical trial of MY9 monoclonal antibody-purged autografts for the treatment of acute myeloid leukemia

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