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HEMOPOIETIC GROWTH AND INHIBITORY FACTORS IN TREATMENT OF MALIGNANCIES

A review

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The clinical use of cytokines is still expanding as the knowledge of beneficial effects as adjunct to cancer treatment is increasing. G-CSF and GM-CSF stimulates hemopoietic recovery after myelosuppressive chemotherapy and enhances engraftment after bone marrow transplantation. New cytokines as IL-1, IL-3, IL-4 and IL-6, are studied in clinical trials and combinations of these with stem cell factor seem promising in ex vivo expansion of stem cells. GM-CSF also have antitumor effects. The most recently discovered hemopoietic growth factor is thrombopoietin, from which probably especially patients with leukemia will benefit.

The effectiveness of chemotherapy is for most human tumors limited by drug resistance and toxicity. Resistance can be overcome to some extent by increased dose, but only at the expense of increased toxicity. For agents where dose is limited primarily by myelosuppression, several strategies are being evaluated to permit dose escalation. Autografting of hematopoietic stem cells from bone marrow or peripheral blood has thus been explored in lymphomas, Hodgkin's disease, breast cancer, and other tumors (1–3). Response rates in these trials tend to be high, but both duration of responses and survival rates vary widely with tumor type, amount of prior therapy and other conditions. Additionally, there has been substantial morbidity and mortality due to myelosuppression and nonhematologic toxicities, such as mucositis, gastrointestinal dysfunction, cardiotoxicity, venoocclusive disease of the liver, and interstitial pneumonitis. High-dose, severely myelosuppressive regimens have therefore been concentrated to specialized transplant centers and reserved for relapsed or poor-prognosis patients. Nonetheless, these trials support the concept that many human tumors have a steep dose–response curve, particularly as regards alkylating agents. Although improvements in supportive care have made autologous marrow (ABMT) or peripheral blood stem cell (PBSC) transplantation a relatively safe procedure, many drawbacks still remain. Significant resources are needed for harvesting and cryopreservation of stem cells from bone marrow or blood, as well as purging of tumor cells from the autograft, when applicable. However, certain types of high-dose chemotherapy can be given without marrow rescue, provided intensive supportive treatment is available. This approach has been most successful with drugs such as cyclophosphamide, which is tolerable in very high doses (4).

High-dose chemotherapy, either with or without stem cell autografting, has not been tested in many types of previously untreated tumors since it poses risks of morbidity and mortality that have generally been considered unacceptable. However, methods to stimulate hematopoiesis might significantly ameliorate some of the toxicities that prevent large-scale investigation and widespread use of such treatment. It is increasingly clear that hematopoietic growth factors can selectively stimulate the production of human blood cells in vivo (5–13). The four major myeloid hematopoietic growth factor (granulocyte-macro-phage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), and

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interleukin-3 (IL-3)) and erythropoietin (Epo) are currently being evaluated in phase I–II trials in cancer patients. Trials with other cytokines with hematopoietic activity, such as IL-1, IL-4, IL-6, IL-8, IL-9 and IL-11, have either begun or are likely to start in the near future. Over the last 3 years, extensive clinical data have been accumulated with G-CSF, GM-CSF and Epo. Despite the many variables in these trials, certain features of the clinical effects of CSFs are becoming apparent. GM-CSF and G-CSF thus demonstrate a capacity to accelerate hematopoietic recovery and reduce the period of dangerous neutropenia. Not surprisingly, GM-CSF and G-CSF are much less effective in patients with reduced marrow reserve, and there is no reason to believe that CSFs can replace stem cell autografting, when truly marrow ablative therapy is used. The leukocytes produced in response to these factors are activated functionally with increased chemotaxy and cytolysis (14). However, in one study (15) GM-CSF-stimulated neutrophils showed decreased mobility into skin windows, raising the possibility that these granulocytes may not migrate optimally into areas of infection or inflammation (9). The known effects of GM-CSF upon macrophage function and the induction of enhanced phagocytic function in soft tissue macrophages may compensate for decreased neutrophil migration. In a clinical study (35) measurement of neutrophil accumulation at skin window sites revealed that rhGM-CSF infusions in 4 patients did not affect neutrophil migration in vivo. Normal responses were obtained even during periods of marked cytopenia.

Despite laboratory evidence that GM-CSF and G-CSF enhance proliferation of marrow progenitor cells of red blood cells (RBC) and platelets in tissue culture, neither GM-CSF nor G-CSF has shown clinically significant effects on production of these cell-lineages until recently, when some data (17) on GM-CSF alone or GM-CSF in combination with IL-3 (18, 19) suggest stimulating effects on platelet progenitor cells.

Toxicities of these CSFs have been acceptable, and effective doses can be given without the pronounced side-effects noted with other biologic agents, such as tumor necrosis factor (TNF) (20) or high-dose IL-2 (21). GM-CSF appears to be somewhat more toxic than G-CSF, perhaps due to GM-CSF-induced priming of monocytes, which enhances secondary release of inflammatory mediators such as TNF and IL-1 (14, 22). Nonetheless, at clinically effective doses, the toxicities of both of these agents appear to be modest.

It is increasingly clear from studies such as that by Gianni et al. (23) that the use of CSFs in oncology patients is likely to be beneficial. It remains to be determined whether CSFs will make very high-dose chemotherapy sufficiently safe so that their use can be widely tested in previously untreated patients in an outpatient setting. Nevertheless, it is reasonable to be optimistic that CSFs may, at the very least, shorten hospital stays, improve the ability to deliver chemotherapy 'on time', reduce the number of septic deaths, and possibly even permit further dose escalation of some drugs such as cyclophosphamide. It is conceivable that CSF administration may reduce the need for marrow autografting when high-dose chemotherapy regimens, that are not lethal to hematopoietic stem cells, are used, or at least considerably reduce the period of neutropenia following auto-transplantation.

Whether increased dose will mean increased cure is another question. Larger studies are needed to prove that dose-intense therapies with CSF support can be administered with acceptable levels of toxicity and costs. Non-hematologic toxicities are likely to be important in redefining the maximum tolerated dose for cytotoxic drugs. It is still not clear if dose escalations made possible by CSFs will result in a clinically significant impact on tumor cell kill in drug-resistant tumors. The prospects of combining CSFs that possess synergistic or non-overlapping activities are exciting, and it is hoped that combinations will be found that accelerate recovery of platelets as well as neutrophils. Also, it is likely that the effects of some of these cytokines will not be limited to the stimulation of blood production. Several effector functions of mature monocytes, granulocytes, T cells, B cells, or natural-killer cells may be enhanced by G-CSF, GM-CSF, M-CSF, IL-3, IL-4, and other factors (14).

Clinical uses of cytokines

Cancer chemotherapy. Myelotoxicity has been the most limiting side-effect of potentially curative cancer chemotherapy. Neutropenia often results in bacterial and secondary fungal infections which are a major cause of morbidity and may be fatal (24). Management of febrile neutropenic patients is costly because of prolonged hospitilization and require antibiotic therapy. G-CSF, GM-CSF or other cytokines may therefore be used to reduce the period of neutropenia following chemotherapy to reduce infectious complications and allow the treatment to be given at full dose. There is also evidence that increasing the dose intensity of chemotherapy may improve the tumor response (25–29).

Hematopoietic growth factors have characteristic effects on the hematopoietic cells. Some factors act exclusively on a particular cell lineage (30). For example, the actions of G-CSF are restricted almost exclusively to the neutrophil lineage, while M-CSF acts upon macrophage precursor cells and erythropoietin upon erythroid precursors. GM-CSF has a broader activity, stimulating progenitors of neutrophils, monocytes and eosinophils. There are also hematopoietic growth factors such as IL-3 that act on the early multipotent progenitor cells, giving rise to cells of both erythroid myeloid lineages. SCF stimulates pluripotential cells, making them more responsive to the effects of

more lineage-restricted growth factors. SCF alone has thus no colony-stimulating activity when incubated with marrow cells, but if G-CSF is added to SCF there is an increase in neutrophil colony formation that is greater than if G-CSF is used alone. Another pluripotent factor under clinical investigation is IL-1 (31). The administration of IL-1α is associated with fever, chills, headache, nausea, vomiting and myalgias. At doses of 0.3 μg/kg or higher, dose-limiting toxicities were frequent, including severe hypotension, myocardial infarction, confusion, severe abdominal pain, and renal insufficiency. On the other hand, i.v. bolus infusion of recombinant human M-CSF at 30 000 $\mu g/m^2$/day or more caused a peculiar syndrome of ocular or periorbital inflammation (32). Recombinant G-CSF (filgrastim) and GM-CSF have been studied most extensively, but no direct comparative studies have been conducted to determine which hematopoietic growth factor provides optimal hematopoietic recovery under different circumstances. However, experimental and clinical data show clear differences between the two agents (33). Studies conducted by Lord (33) have shown that G-CSF produces a greater and more rapid increase in neutrophil count than GM-CSF. Clinical studies have shown that recombinant G-CSF and recombinant GM-CSF successfully accelerate neutrophil recovery following myeloablative therapy and BMT (34–36). A decrease of the neutropenia period was associated with reduced use of parenteral antibiotics and days of hospitalization. GM-CSF treatment was associated with improved survival in patients with poor graft function after transplantation (35). No studies have been conducted to determine whether G-CSF or GM-CSF is the more effective agent following high-dose chemotherapy and BMT. Neither G-CSF nor GM-CSF stimulate platelet recovery and so alternative strategies are required when thrombocytopenia is dose limiting. Recombinant IL-3 has been studied for its activity on megakaryocytes, but demonstrated in early clinical studies only weak effects on platelet counts (37). Investigators also look at the combination of two or more growth factors, which may produce a synergistic response and hence promote both platelet and neutrophil recovery. For example, the effect of GM-CSF plus IL-3 has been investigated (38). The results showed the neutrophil recovery rate was not accelerated more in these patients compared to patients receiving GM-CSF only. Combinations of hematopoietic growth factors may also produce unexpected effects and their clinical use must therefore be regarded as experimental. For example, a recent report (39) showed that a combination of GM-CSF and IL-3 delayed platelet recovery after ABMT. Such observations may perhaps also be relevant to clinical trials with the interesting IL-3/GM-CSF fusion molecule known as PIXY-321. Moreover, the sequential use of GM-CSF and IL-3 could theoretically be counterproductive because the increased differentiation of the precursor pool induced by GM-CSF might reduce the pool of IL-3 sensitive cells. A combination of a myeloid growth factor and a true megakaryocyte CSF might have clinical relevance but this has not yet been unequivocally proven. The most promising technique available to accelerate platelet recovery is the use of PBSC mobilized by an hematopoietic growth factor. It is surprising that many different types of agents can considerably increase the number of circulating hematopoietic progenitors: cytotoxic drugs, high-dose corticosteroids, some antibiotics, folinic acid, some bacterial compounds, and several growth factors or cytokines (G-CSF, GM-CSF, IL-3, IL-1, IL-2, SCF, erythropoietin and IFN-α-2b). Early dose-ranging trials provided firm evidnece that G-CSF and GM-CSF can support cell counts after chemotherapy (10, 40). Some of these studies with GM-CSF have also given indications of other benefits, as discussed later. Antman et al. (10) achieved an improved tumor response rate of 79% compared to 52% in a previous study of historical controls. Nine out of 14 patients with non-Hodgkin lymphoma (NHL) achieved complete remission compared to 2 out of 14 not treated with GM-CSF. In a study by Herrmann et al. (41) duration of hospital stay and need of antibiotics were also reduced. Ho et al. (17) reported shorter periods of thrombocytopenia and reduced rates of infection and stomatitis. Using continuous infusion of GM-CSF duration of thrombocytopenia and neutropenia induced by melphalan (120 mg/m^2), was at least as short as those reported in a historical series receiving autologous BMT (42, 43). Edmonson et al. (44) reported that they could overcome the dose-limiting thrombocytopenia by rescheduling the treatment program for GM-CSF. Interesting attempts have been made to protect myeloid cells from the effects of cell cycle-specific agents. This was originally proposed by Aglietta et al. (45, 46) from a study of the cell kinetics of the response to GM-CSF. This study suggested that a short period of administration just prior to chemotherapy would enable the cytotoxic agents to be given on schedule. If the GM-CSF was stopped 24–28 h before the chemotherapy, the normal bone marrow cells could be put out of cycle and protected from toxicity of cell cycle-specific drugs. This has recently been confirmed in patients with inoperable metastatic sarcoma, where myeloprotection could be significantly enhanced by optimising timing and schedule of GM-CSF and chemotherapy (47).

Bone marrow and peripheral blood stem cell transplantation. Animal and human studies suggest that escalating the dose of cytotoxic therapy may give improved tumor response (25–29, 48). However, the myelotoxicity of aggressive chemo/radiotherapy requires support by peripheral blood stem cells (PBSC), or allogeneic or autologous BMT to restore normal marrow function. Despite this procedure, recovery of neutrophil counts takes approximately 2–3 weeks (49) during which time the patient remains at considerable risk of bacterial and fungal infections.

Thrombocytopenia may persist for long periods requiring repeated platelet transfusions. The high morbidity rates, 5–10% infective deaths (50) and high costs have limited the use of BMT. The use of hematopoietic growth factors has clearly reduced morbidity in the BMT setting, but whether these agents will also improve survival remains to be seen. However, despite the use of hematopoietic growth factors, BMT will remain a difficult procedure restricted to specialized centers. Brandt et al. (9) administered GM-CSF (2.0–32.0 μg/kg per day) i.v. for 14 days after BMT producing faster engraftment compared to historical controls. No differences in platelet counts were reported. In a separate study i.v. daily doses of 15–240 μg/m^2 to 15 patients with lymphoid malignancies (51) produced earlier neutrophil and platelet recovery at doses above 60 μg/m^2 per day with fewer days of fever and earlier discharge from hospital. Initial studies indicated that GM-CSF can promote the myeloid recovery achieved with BMT (9, 51–53). Preliminary reports of 6 randomized double-blind trials have provided more convincing evidence of significantly faster recovery of the neutrophil counts with GM-CSF from a total of 546 patients (54–59). These studies, which enrolled patients with a variety of malignant disorders, have revealed other benefits. Compared with patients receiving placebo, those given GM-CSF had experienced less infections with reduced need of antibiotics and other hospital resources. Experience from 10 patients indicated that the severity of graft versus host disease may be ameliorated by GM-CSF (60) and that depressed neutrophil function may be restored (61). It has been suggested that BMT should be limited only to patients with chemosensitive disease in first remission, but there are obvious problems with this approach; because of costs and toxicity, BMT has been used primarily when standard therapies are unikely to produce durable remissions. If conventional therapies improve (by new drugs or new strategies) it will be difficult to justify BMT (62). PBSC as an alternative source of hematopoietic progenitor cells may be used in conjunction with, or instead of, BMT to support the delivery of intensive chemotherapy (63). The advantages of PBSC over BMT are lower procedure-related morbidity, faster recovery of neutrophils and platelets, and an increase of immunocompetent cells in the graft. The comparative disadvantages of PBSC include the varying efficacy of the harvesting procedure possibly related to previous extensive chemotherapy or radiotherapy, the laborious procedure and the fact that the long-term permanency of engraftment has not been fully evaluated.

Before the use of hematopoietic growth factors, stimulation of the progenitor cell pool was only feasible by administration of a chemotherapeutic agent (64). However, stimulation of PBSC by a marrow-toxic drug has a number of disadvantages, as noted above. Hematopoietic growth factors produce a more consistent and prolonged increase in PBSC and do not produce the severe side-effects associated with chemotherapy (63). Some investigators have, nevertheless, used cytotoxic agents plus hematopoietic growth factors to maximize the yield of PBSC while delivering standard-dose chemotherapy early in the treatment course, prior to the initiation of dose-intensive treatment (65). In several pilot studies, PBSC mobilized by hematopoietic growth factors has been used of high-dose chemotherapy (66–68). These studies have not yet identified suitable doses and schedules for the different growth factors, but they have clearly shown the clinical efficacy of PBSC in restoring hematopoiesis. For example, Sheridan et al. (66) treated patients with high-dose chemotherapy followed by BMT and daily administration of recombinant human G-CSF (filgrastim). One series of patients also received filgrastrim-mobilized PBSC collected prior to high-dose chemotherapy. The addition of PBSC was associated with a significant acceleration in platelet recovery compared with BMT plus filgrastim. Neutrophil recovery was similar in patients with and without PBSC, but in both groups recovery was faster than in historical controls who received BMT only. PBSC mobilized by G-CSF and GM-CSF may be used without bone marrow to restore hematopoiesis (69, 70). Although strict comparative studies have not been published, it appears that mobilized PBSC alone are as effective as mobilized PBSC plus BMT in accelerating neutrophil and platelet count recovery (69). G-CSF-mobilized PBSC have been used to allow the delivery of high-dose therapy in children, in whom preserving dose intensity is of paramount importance owing to the potential curability of many pediatric tumors (71). The study included seven children with advanced neuroblastoma or NHL. PBSC collected after stimulation with chemotherapy and G-CSF (filgrastim) were reinfused after myeloablative chemo-radiotherapy. Hematopoietic reconstitution appeared to be stable over the observation period of up to 22 months. Clearly, the use of PBSC is a promising approach for delivering high-dose chemotherapy more safely and, hopefully, more effectively. However, the technique remains experimental and further studies are required. The long-term effects of using PBSC without BMT must be determined before the clinical use of PBSC is confirmed. Furthermore, the need for additional hematopoietic growth factor therapy after PBSC transplantation should be evaluated in prospective studies. If the initial encouraging results are confirmed, the use of PBSC may eventually supersede BMT and thus make high-dose chemotherapy a realistic option for a larger number of patients. Another important aim of clinical research would be to improve the PBSC yield and minimize the number of leukapheresis procedures required. It should be feasible to achieve hematological recovery using PBSC collected from a single leukapheresis procedure instead of the three or so procedures currently used (72). It has recently been documented (73) that many patients with high-risk breast cancer, small cell and non-small cell lung

cancer, have circulating tumor cells which should imply a substantial risk of concomitant tumor cell recruitment upon mobilization of PBSCs. The biologic and clinical significance of this finding is unknown at present.

Expansion of hematopoietic cells ex vivo. An alternative to the use of hematopoietic growth factors in vivo is their use ex vivo to amplify blood progenitor cells, which can then be reinfused to the patient. Ideally, one would like to amplify not only the committed progenitors cells but also the true pluripotent stem cells that might then be used for grafting following complete myeloablation. Ex vivo expanded cells could save harvesting time and effort (much smaller volumes of autologous marrow would be required), and might also reduce the required infusion dose. Moreover, these stem cells could be ideal targets for some gene therapy experiments, or one might attempt to selectively amplify peripheral blood cells responsible for graft-versus-leukemia effects rather than GVHD from allogeneic donors. Although it is clear that the bone marrow stroma which consists of fibroblast-like cells, adipocytes, endothelial cells and other less well chracterized cells, is important for survival, proliferation and differentiation of hematopoietic cells, it is unclear whether any of these functions can be completely replaced by the use in vitro of early-acting growth factors (74). In 1990, three different groups reported cloning of the product of the Steel gene locus, which turned out to be the ligand of the c-kit proto-oncogene. This protein was called stem cell factor (SCF), kit ligand or mast cell growth factor (MCGF), and is synergistic with multiple growth factors in clonogenic assays of murine and human bone marrow, and, in particular, with IL-3, G-CSF, GM-CSF (or IL-3/GM-CSF fusion protein), IL-6, IL-11 and IL-1. In a recent report, Muench et al. (75) described successful ex vivo expansion of hematopoietic progenitors in murine short-term suspension cultures using several combinations of growth factors with SCF. IL-1 plus SCF expanded progenitors most effectively, and these progenitors accelerated the recovery of peripheral blood leukocytes, platelets and erythrocytes in lethally irradiated mice. The feasibility of amplifying ex vivo, progenitor and pluripotent stem cell collected by leukapheresis and subsequently exposed to a variety of growth factors in vitro, is currently being explored by several groups. Interesting activity has been reported for the combination of SCF, IL-1, IL-6, IL-3 and erythropoietin (76). To survive in vitro it seems likely, however (at least on theoretical grounds) that a true pluripotent stem cell will require, not only the appropriate combination of hematopoietic growth factors, but also the appropriate stromal microenvironment.

Mobilization and harvest for PBSC. The harvest of PBSC cells for transplantation by leukapheresis of an unstimulated donor can easily require more than 10 sessions of pheresis. Stimulation with a cytotoxic agent or a CSF can lead to sufficient numbers of circulating PBSC to be used for transplantation after just one to five leukapheresis sessions. It is unclear which cytotoxic drug(s) is best for mobilization, but some (e.g. the nitrosoureas) are potentially so damaging to stem cells that the clonogenic ability of PBSC mobilized with these agents can be significantly impaired. Many cytotoxics have been used to mobilize PBSC, either alone or in combination. They include high-dose single agents (such as cyclophosphamide 3–7 g/m^2, or etoposide 2 g/m^2), or combination therapy at conventional doses (e.g. after CHOP-type, CAF, FEC or ICE-type chemotherapies) or at higher than conventional doses. G-CSF (filgrastim), GM-CSF and IL-3 have all been used to generate autologous PBSC for transplantation use. For example, Sheridan et al. (66) gave 17 patients with non-myeloid malignancies filgrastim, 12 μg/kg/day for 6 days and collected progenitor cells by leukapheresis on days 5, 6 and 7. Granulocyte-macrophage progenitors increased 58-fold and erythroid progenitors increased 24-fold. Another strategy to generate PBSC is to give the hematopoietic growth factor following treatment with cytotoxic agents, such as cyclophosphamide, to enhance the chemotherapy-induced increase in PBSC (23). This approach has been investigated in pilot studies using G-CSF (filgrastim) (72) glycosylated G-CSF (lenograstim) (77), GM-CSF and IL-3 (23, 65). At present, however, there are no direct comparative data to indicate which is the most effective hematopoietic growth factors in terms of numbers of PBSC collected or rate of hematological recovery following transplantation.

Myelodysplastic syndrome (MDS). Early studies established dose-related increase in leukocyte count with GM-CSF (78, 79) and that proliferation of the blast cells could be controlled or reduced (80). Firm evidence of GM-CSF's ability to repair myelopoiesis in MDS has emerged in three randomized trials. Schuster et al. (81) randomized 133 patients to either GM-CSF (3 μ/kg per day s.c.) or observation, over a 90-day period. The neutrophil counts of observation patients remained at the baseline values of around 0.6×10^9/l while patients receiving GM-CSF had significant increase to around 3.8×10^9/l. There were also increases in monocytes, eosinophils and lymphocytes and fewer major infections (15 vs. 33%) in patients treated with GM-CSF. The two other studies differentiated patients according to their risk of transformation to leukemia. In low-risk patients, encouraging response rates of 60–70% have been reported which were not proportional to the starting counts (82) or the dose of GM-CSF. In high-risk patients, who received low-dose cytarabine with concomitant or subsequent administration of GM-CSF, 46% patients were classified as partial responders or better (83). Further, some improvement in bone marrow function was documented after several weeks' follow-up. Two of these studies monitored transformation and found no evidence that GM-CSF promoted progression to leukemia. As a differentiation-inducing agent vitamin D has been used to

increase erythropoiesis and platelets counts in MDS. GM-CSF and vitamin D were combined in cytopenic, symptomatic MDS (84) in an open pilot study. A hematologic response other than raised neutrophil counts in 30% of patients suggests an additive effect of these two agents in MDS.

Myeloid leukemia. Studies from the Toronto group have shown that the clonogenic blast cells in acute myeloid leukemia can proliferate and expand in response to IL-3, GM-CSF or G-CSF (alone, or in combination) and at least some times undergo preferential differentiation in response to M-CSF (85). At first sight, these data might preclude a role for IL-3, GM-CSF or G-CSF in the treatment of AML. However, these and other studies (86) suggest that these hematopoietic growth factors may be particularly useful for recruiting quiescent leukemic stem cells into cell cycle and thus rendering them sensitive to the cytotoxic effects of cycle-specific drugs (87). Obviously, the dose and scheduling of the hematopoietic growth factors will need to be tailored to the disease, but an added 'bonus' could well be the differentiation-inducing activity of these growth factors. In this context also, it should be noted that this approach will only be successful if normal stem cells are not responsive to the hematopoietic growth factors and, therefore, not recruited into cell cycle and thus sensitized to the effects of the chemotherapeutic agents.

Antitumor activity. The functional enhancement of macrophage and monocytemediated antibody-dependent cell cytotoxicity has led some investigators to suggest that GM-CSF may have antitumor activity. Supporting evidence comes from in vitro observations of GM-CSF-stimulated human and murine bone marrow cultures, which showed significant lysis of two tumor cell lines (88). This antitumor effect has also been observed in vivo in two mouse models of cyclophosphamide and TBI therapy followed by BMT (88). Ruff et al. (89) reported that GM-CSF inhibited the proliferation of small cell lung cancer (SCLC) cell lines when given at high doses. Likewise in a randomized study (90) mice implanted with Lewis lung sarcoma were treated with GM-CSF 1 μg/day or used as controls. No cytotoxic drugs were used. After 10 days the tumor volumes were significantly lower in the GM-CSF treated mice than in the controls. In a parallel study (90), peritoneal macrophages were harvested from mice and studied (with or without GM-CSF co-culture 50 ng/ml per 10^6 cells) for macrophage antitumor mechanisms including oxygen-free radical production, nitric oxide release and non-opsonized phagocytic function. GM-CSF significantly up-regulated all the mechanisms studied. However, other conflicting results have been published (91–93), which will be commented on later. The demonstration of receptors for G-CSF and GM-CSF on SCLC cells may be relevant in this respect, although transduction to a functional intracellular signal should also be sought (94). Morstyn & Burgess (95) have proposed that an antitumor effect is unlikely to be achieved with systemic administration but could be feasible for isolated tumors where local administration of GM-CSF might be more effective. However, this does not follow from observations in 20 patients (Scarffe: personal communication) undergoing a phase I trial of GM-CSF. These patients received infusions on days 1–10 and 21–30, with infusions every other day from days 31–50 at doses ranging from 0.3 to 60 μg/kg per day. Regular monitoring of evaluable sites revealed stabilization of disease in 7 patients up to a minimum of 70 days from the start of the study. One patient with a heavily pretreated liposarcoma experienced a significant reduction ($>$ 50%) of the tumor volume. Metcalf (96) has suggested that the degree of stimulation and suppression in leukemia may vary according to the leukemic population and that in vitro screening assays should be developed to identify patients in which there is antileukemic potential for GM-CSF. In vitro culture of leukemic cell lines has suggested that combination of GM-CSF with other cytokines will inhibit their growth (97). The development of leukemia inhibitory factor (LIF) and its possible synergistic actions with GM-CSF may have important consequences in this connection (98). Recent data from a randomized double-blind placebo-controlled phase III study of GM-CSF as adjunct to induction-chemotherapy of aggressive NHL (99), revealed that 31/45 (69%) high-risk patients treated with GM-CSF achieved CR, as compared to 25/52 (48%) placebo-patients. However, the dose intensity was somewhat higher in the GM-CSF treated patients indicating that the higher response-rate was not solely a matter of cytokine tumoricidal effect. Analogous observations were made by the author in an accepted but yet unpublished prospective study where 20 patients with chemotherapy-treated metastatic breast cancer were randomized to receive or not receive GM-CSF. In the cytokine-arm the response rate was 64% compared to 28.5% in the control-arm with equal dose intensity. Fifteen patients with various advanced soft tissue sarcomas were treated with cyclic ifosfamide/doxorubicin and cisplatin with or without mitomycin in combination with GM-CSF (100) and in three progressively more intensive cytotoxic drug regimens GM-CSF was given 4 days prior to chemotherapy as well (101). Complete tumor regression occurred in 6 patients. In addition to the unexpected frequency of durable complete tumor regressions, three other patients experienced partial remission. Perhaps the intensive use of GM-CSF enhanced the therapy of these patients, but further studies are of course needed before any definite conclusion can be drawn. Hypothetically, molgramostim (GM-CSF) may increase tumor cell kill by stimulation of antitumor immunity. Although such an immunostimulatory role for subcutaneous GM-CSF awaits direct experimental proof, this idea logically follows the observation of enhanced tumor destruction in mice bearing tumor cells caused to secrete GM-CSF by transfection with its gene (102). Can

intensive subcutaneous treatment with GM-CSF mimic the effects of intratumoral secretion of this agent? A report (103) using subcutaneously injected gelatin-chondroitin sulfate microspheres in mice suggests that it might.

Anti-infective properties. The ability of GM-CSF to enhance the funciton of neutrophil, eosinophil and the monocyte-macrophage lineages has suggested its use in promoting host defence. Two reports have provided evidence which supports a clinical role for GM-CSF in promoting the response to acute infections. The earliest of these described how GM-CSF can reduce the proportion of cultured peritoneal macrophages collected from mice infected with Leishmania tropica (104). In this study there was a continuous decrease in the percentage of infected cells reaching less than 10% on day 4 compared to 30% in controls and there was an indication of increased killing of these parasites. A later study demonstrated that GM-CSF could inhibit the replication of Trypanosoma cruzi by activation of macrophages in both human and murine cultures using the homologous cytokine (105). This was mirrored by increases in the ability to release hydrogen peroxide. Increase in circulating CSFs certainly seems to be a normal component of the biological response to bacterial infection (106). In a study of endogenous hematopoietic growth factors in neutropenia and sepsis (107), G-CSF, IL-6 and M-CSF levels were significantly elevated in sepsis. In contrast, GM-CSF levels were not elevated. Elevated G-CSF and IL-6 levels normalized rapidly (hours-days) with the restriction of infection, whereas M-CSF concentrations remained elevated for up to 10 days. Cytokine levels remained elevated in septic neutropenic patients, who did not recover. However, Jensen et al. (108) reported that GM-CSF had no effect on the antibacterial effect of peripheral blood monocytes and pulmonary macrophages in vitro where an effect of gamma interferon could be demonstrated. Administration of G-CSF in combination with appropriate antibiotics afforded a dose-related inhibition of death from infection in mice after cyclophosphamide injection (109). More successful results have been reported for fungal infections (110). GM-CSF seems to stimulate the fungicidal activity of human monocytes in vitro and this is associated with enhanced production of superoxide (111). In a non-randomized study, 8 patients with disseminated fungal infection treated with amphotericin B also received GM-CSF (112); four were completely cured of fungal infection and two had a partial response. Many clinical studies of GM-CSF now assess infection and secondary endpoints related to it. The pattern emerging is that there are significant reductions of serious infections and marked savings in antibiotic use, hospitalization and support measures such as isolation (17, 41). However, it may be difficult to demonstrate a prophylactic anti-infective effect by direct comparisons with established antibiotic regimens. A recent example of this is a small study of patients receiving intensive chemotherapy for lymphoma. GM-CSF 125 $\mu g/m^2$ i.v. over 6 h, days 6–21, was compared with prophylactic antibiotics (days 6–21). The number of documented infections was higher with GM-CSF which, coupled with higher requirements for red biood cels and platelets, led to early termination of the study (113).

Potential clinical problems with the hematopoietic growth factors

Recruitment of stem cells. The stimulation of proliferation and differentiation of primitive multipotent 'stem' cells using IL-3 or various combinations of otherwise lineage-restricted growth (such as G-CSF and GM-CSF) in vitro, raised the possibility that in vivo treatment with these agents may lead to premature exhaustion of the hematopoietic system due to excess recruitment of stem cells. So far, no experimental or clinical evidence has emerged to support this possibility. One reason for this may be that the effects observed in vitro represent an aberrant response of hematopoietic cells to the hematopoietic growth factors. A more likely possibility, however, is that, within the bone marrow, the cellular environment exerts a restraining influence upon the hematopoietic cells. Consider for example, the effects of G-CSF: in vitro, this can synergise with a variety of other cytokines and promote the development of multiple cell lineages (114, 115); on its own, however, it is a relatively poor stimulus for progenitor cell development and recruits mainly neutrophil development from precursor cells. This is also the response elicited in vivo, where the most dramatic effects are seen on production of neutrophils (6, 116, 117). This suggests that the other cytokines, with which G-CSF can establish synergistic interactions, are not 'freely' available in vivo—supporting the concept that they are produced/sequestered by the marrow stromal cells and are localized to discrete sites. Support for this view has come from recent studies using long-term human marrow cultures, where treatment with recombinant human G-CSF had a modest effect upon production of neutrophils, but little or no effect upon the production of multipotent or lineage-restricted progenitor cells for the other cell lineages (118). Similar arguments can be raised for GM-CSF and for IL-3 where no evidence has emerged (in vivo or in vitro) suggesting that these agents interfere with the proliferative capacity of the multipotent cells (118). At which developmental stages then, do these agents exert their effect in vivo? Perhaps the most comprehensive study has been done with G-CSF, where labelling studies have clearly shown that the major effect of treatment is an increase in the number of cell divisions occurring in the neutrophil precursors (117). In combination with a reduced marrow transit time, this means that more mature cells are released earlier into the circulation. However, the number of divisions elicited by G-CSF, necessary to achieve this effect, is only in the order of three

to four. The important point is that this is a fairly modest response and certainly does not require 'input' from earlier (multipotent) cells to achieve the effect. It should be stressed, however, that while treatment with individual growth factors has not apparently led to depletion of multipotent 'stem cells', combination treatments using two or more of the hematopoietic growth factors may have greater than additive effects and their use in this way should be approached cautiously.

The question of lineage competition. During treatment of mice with G-CSF, there is a marked supression of erythropoiesis is the bone marrow (119). Although in normal mice this is compensated for by the induced erythropoietic activity in the spleen (119, 120) long-term treatment with G-CSF eventually manifests as a severe anemia. The mechanisms underlying this effect are unclear, but may represent a spatial restriction due to the increase in granulopoiesis, or may represent competition at the level of stem cell differentiation and lineage commitment. It should be stressed, however, that the hematopoietic system of mice is different from man in that mice have little or no yellow marrow, i.e. there is little or no room for expansion of medullary hematopoieses. Also, the suppression of erythropoiesis seen in mice treated with G-CSF has not been observed during treatment with GM-CSF (121). Furthermore, clinical data available so far have not shown a consistent anemia or thrombocytopenia developing in patients treated with hematopoietic growth factors. Thus, although 'lineage competition' may be a theoretical consequence of treatment with hematopoietic growth factors, it does not yet appear to represent a clinically significant hazard.

Effect of long-term treatment with growth factors. Following the discovery that GM-CSF transgenic mice developed a lethal syndrome (122), questions were raised as to the long-term effects of hematopoietic growth factor treatments. Continuous treatment of primates or mice with GM-CSF or G-CSF, however, has not shown life-threatening effects and the transgenic model cited above is probably not of clinical significance. Perhaps the lethal syndrome in these mice is related to the anomalous constitutive production of GM-CSF in various tissues or reflects the extremely high levels of circulating growth factors. Whatever the reason, it is worth noting that maintenance of high levels circulating G-CSF in mice (through transfer of cells carrying a retrovirus expressing the gene for G-CSF) does not lead to death of the animals nor to pathological alterations in the tissues (123). The clinical effects of continuous, long-term treatment with hematopoietic growth factors need to be further studied, although patients with chronic neutropenia have received G-CSF for up to two years without loss of effect.

Other target cells for hematopoietic growth factors, stimulation of proliferation of malignant cells

Data suggest that the majority (perhaps all) of the lymphoid malignancies do not express receptors for the myeloid cell hematopoietic growth factors (30, 124). Indeed, several clinical trials are currently taking place where hematopoietic growth factors are being used to enhance regeneration of granulocytes following treatment of Hodgkin's and non-Hodgkin's lymphomas with chemotherapy or bone marrow transplantation. Thus far, stimulation of growth of lyphoid tumors or increased relapse rates have been not reported following growth factor treatment. Similarly, no evidence suggests so far that G-CSF or GM-CSF enhance the in vivo growth of solid tumor cells, although patients with a variety of tumor types have been treated. Some hematopoietic growth factors may have an antitumor effect, but convincing data are so far lacking (125). The in vitro data, however, are more ambivalent in that cell lines derived from a variety of solid tumors show significantly enhanced proliferative response when exposed to GM-CSF and/or G-CSF (126, 127). Also, primary cultures of normal endothelial cells show enhanced proliferation and migration in response to these growth factors (128). It is not clear, however, whether normal endothelial cells respond in vivo to GM-CSF, G-CSF or the other myeloid hematopoietic growth factors: neo-vascularization has not been reported and no data from animal studies have shown an effect of these agents on angiogenesis. Some tumors cell lines have proliferated in response to GM-CSF (91, 127–129). However, stimulation of the growth of non-hematological tumors is not normally seen in the presence of serum and may therefore not be clinically relevant. Where growth has been observed, it has usually been modest. Screening of GM-CSF in the human tumor clonogenic assays has provided no consistent evidence of stimulation of the growth of fresh tumor explants (130). GM-CSF can stimulate the proliferation of osteogenic sarcoma cell lines, a breast cancer cell line and fibroblast precursors, as measured by ^{3}H-thymidine incorporation (127). Receptors for GM-CSF and G-CSF have been reported in small cell lung cancer cell lines (91) and GM-CSF has been reported to stimulate, inhibit or have no effect on cell proliferation. Clinical studies of G-CSF and GM-CSF in advanced cancer have usually found no stimulation of tumor growth. It seems as if the CSFs do not stimulate tumor cell proliferation significantly and only a small proportion of tumors are likely to exhibit CSF receptors.

Characterization of new cytokines

Stem cell factor. A factor which preferentially increases the proliferation of primitive hematopoietic progenitor cells has been identified and cloned (131); it was called

stem cell factor (SCF), but is also known as c-kit ligand and mast cell growth factor (MGF). A protein acting upon early hematopoietic progenitor cells was purified from Buffalo rat liver cells and its amino acid sequence determined. Subsequently the gene for this protein was cloned and expressed in *Escherichia coli*. By genetic probes based on the rat sequence, clones of the human SCF gene could then be isolated. This gene was expressed in microbial and mammalian hosts which produce recombinant human SCF. The SCF receptor has been identified as c-kit (a tyrosine kinase receptor) (132). Studies in vitro showed that both rat and human SCF act synergistically with other growth factors in induce proliferation of purified stem cells. In vivo, both rat and human proteins increase the number of primitive hematopoietic progenitors. In one study, rat SCF was administered to normal mice for 7 days. SCF produced an increase in the absolute number of hematopoietic stem cells. Furthermore, both bone marrow and spleen stem cell count increased, most dramatically in the spleen (50–100-fold). The next series of experiments with SCF were performed in baboons (133). In animals treated with SCF, the number of bone marrow and peripheral blood multilineage hematopoietic cells increased between 2- and 5-fold. SCF induced dramatic and sustained increases in the absolute number of colony forming cells in peripheral blood and bone marrow (131). Increases of 100–1000-fold were seen in CFU-GM (the progenitor for granulocytes and macrophages) and BFU-E (the progenitor of erythrocytes). The multilineage progenitor CFU-Mix, which gives rise to erythrocytes, megakaryocytes, monocytes and granulocytes, was increased more than 1000-fold following treatment with SCF. Peripheral blood counts returned to baseline within 7 days after stopping SCF infusions. These promising pre-clinical data suggest that SCF, alone or in combination with a later-acting factor, such as granulocyte colony stimulating factor (G-CSF), may be useful in the treatment of myelosuppression.

M-CSF. Although M-CSF supports monocyte-macrophage progenitor development in vitro in the murine system, its potentiation of macrophage colony formation from human bone marrow is relatively weak. M-CSF increases monocyte and macrophage number, increases macrophage antitumor (both antibody directed and antibody independent) and antimicrobial activity, and stimulates the secondary release of other cytokines, including G-CSF, GM-CSF, IL-1, TNF and interferon (134, 135). These actions may potentially enable M-CSF to prime monocytes and macrophages to combat infections. M-CSF exhibits the capacity to prime murine peritoneal macrophages for killing of TU-5 sarcoma cell line in vitro (136) and induces antibody independent monocyte tumoricidal activity against a murine fibrosarcoma cell line (137).

IL-4. In vitro studies have shown that IL-4 has multiple effects on cells of hematopoietic and non-hematopoietic origin (138). IL-4 has been administered intravenously to monkeys by either continuous infusion or bolus infection. In reports, IL-4 significantly increased white blood cell count, especially neutrophils (139). In addition, at low doses, IL-4 stimulated the phagocytic activity of peripheral blood granulocytes without altering white blood cell count substantially (139). Experiments in mice have shown a possible antitumor effect of IL-4. Recombinant murine IL-4 was administered to tumor-bearing mice and produced a dramatic inhibition of growth of epithelial tumors. It was suggested that the antitumor effect of IL-4 was mediated through the host immune response. Furthermore, in a phase I trial of IL-4 there is evidence of antitumor activity in patients with lymphoid malignancies (140). These data suggest a broad range of clinical applications for IL-4 in the treatment of cancer and the immunocompromised patient.

Other new hemopoietic growth factors. IL-6 has shown thrombopoietic activity in animal models and phase I clinical trials (141). Data on the possible therapeutic potential of IL-6 will emerge shortly. Promising cytokines undergoing preclinical development at present time are IL-11 (possible thrombopoietic activity), IL-8, IL-9, macrophage-inflammatory protein (MIP)-1α (possible stem cell protection during chemotherapy) (142) and a GM-CSF/IL-3 fusion protein (possible combining or potentiating the effects of GM-CSF and IL-3 when given alone) (143).

The search for a platelet growth factor

Thrombopoietins. Although several cytokines have some activity on megakaryo poiesis and thrombopoiesis their effects are weak or only seen in combination with other factors. In vitro studies have shown thrombopoietic activity for the following factors in increasing order of potency: GM-CSF; (IL-6); IL-3; GM-CSF + IL-3; SCF; SCF + GM-CSF; SCF + IL-3; SCF + IL-3 + GM-CSF (143). It has also been reported from primate studies that leukemia inhibitory factor (LIF) produces an increase in platelet count of a similar magnitude as IL-6, but a combination of these two cytokines is not additive. SCF alone did not increase platelet count in monkeys and the combination of SCF with IL-6 was no more effective than IL-6 alone (144).

Meg-CSF. A factor with megakaryopoietic activity has been isolated from a thrombopenic patient with TAR syndrome. This factor supported the growth of CFU-Meg (megakaryocyte progenitors) (145), but not CFU-E or BFU-E (erythroid precursors). Other factors tested in the same assays were not active in supporting the growth of CFU-Meg. Sources for the production of this candidate 'Meg-CSF' are now searched. It has recently been shown (146) that the proto-oncongene c-mpl encodes a protein, whose sequence shares striking homologies with members of the highly conserved hematopoietin receptor super-

family. The data provide the first evidences that c-mpl is involved in megakaryocytopoiesis. In addition, the results raise the possibility that this proto-oncogene encodes the receptor for a new cytokine specifically regulating thrombocytopoiesis.

IL-1. Interleukin-1 (IL-1) is an earlier acting multilineage growth factor reported to increase thrombocytopoiesis. Jakubowksi, New York, USA, has presented the results of a study of IL-1 in 19 patients with gastro-intestinal carcinoma receiving myelosuppressive doses of 5-fluorouracil (personal communication). Patients received for one month each, IL-1 alone, 5-FU alone, then 5-FU + IL-1. Twelve patients completed all cycles of therapy. IL-1 was associated with a transient fall in leukocyte count mainly due to lymphocytopenia, followed by an increase attributable to neutropenia. At the highest dose of IL-1, the absolute neutrophil count increased by almost 5-fold. Platelet count also increased reaching a peak after 2–3 weeks. The dose-limiting toxicity of IL-1 was hypotension. Other side-effects included chills, rigors, fever, musculoskeletal pain and phlebitis. The effects of IL-1 are due at least in part to the induction of other cytokines.

Future prospects

Clinical use of hematopoietic growth factors. The clinical studies with G-CSF, GM-CSF and erythropoietin, (and preliminary work also with IL-3) have given encouraging results and suggest a widespread use in the management of patients with malignant disease, various hematopoietic disorders and following bone marrow transplantation. So far, however, only modest effects upon platelet regeneration have been observed and, for this reason, there is a great deal of interest in defining the cytokines necessary for megakryocyte development. One possibility is 'thrombopoietin'—but the characteristics and mode of action of this molecule are still under investigation (147). In the shorter term, however, the priming and/or synergistic effects of combinations of growth factors are under consideration and phase I/II trials are already in progress to determine the efficacy of sequential or concomitant treatment of patients with IL-3 in combination with another hematopoietic growth factor to hasten platelet regeneration. Combination therapy may also be useful for management of patients after bone marrow transplantation and for treatment of patients with aplastic anemia and MDS. With respect to the use of hematopoietic growth factors for marrow rescue during chemotherapy high-dose for malignant disease, one exciting possibility is to use peripheral blood cells harvested during growth factor treatment. It is known that both G-CSF and GM-CSF can mobilize primitive hematopoietic cells into the circulation—and studies have shown that blood cells harvested from mice treated with G-CSF are as good as (if not better than) bone marrow cells for reconstituting and maintaining hematopoiesis when transferred into irradiated syngeneic mice (119). This, together with data showing that GM-CSF can also recruit primitive cells into the blood and that these (combined with marrow cells) raises the possibility of using such cells routinely for marrow rescue during dose intensification. Perhaps hematopoietic growth factors could also be used to treat potentially related or unrelated donors so that blood (rather than bone marrow) from these individuals could be used as a source of allogeneic stem cells for grafting.

Hematopoietic growth inhibitory molecules. So far, most growth factor research has been concentrated on growth stimulatory molecules. But how is homeostasis maintained during normal steady-state hematopoiesis? What determines the balance between stem cell self-renewal and stem cell differentiation? What determines the size of the various cell populations in a regenerating hematopoietic system and how is proliferation 'switched off' when the optimal size has been reached? In the simplest case, proliferation of the hematopoietic cells could be regulated by modulation of the production or availability of growth stimulatory molecules. Mathematical models suggest, however, that such a simple control system would result in fairly dramatic oscillations in the producton of mature blood cells (148). A more likely scenario is that proliferation is regulated, through a variety of feedback loops, by growth inhibitory molecules acting locally. One candidate is TGF-beta (149). Hampson et al. (150) and others (151–154) have shown that the proliferation of oligopotent and multipotent cells, in response to a variety of hematopoietic growth factors, can be inhibited in the presence of TGF-beta. The more mature, developmentally restricted progenitor cells show more resistance to the growth inhibitory effects of this molecule, while the immediate precursor cells are inhibited little, if at all. In other words, the growth inhibitory effects of TGF-beta are differentiation-linked. Significantly, the effects of TGF-beta are cytostatic rather than cytotoxic and the concentration of TGF-beta required to elicit a response is well within the likely physiological concentration of this cytokine within the environment of the bone marrow. Other growth inhibitory molecules have also been described for hematopoietic cells. One of these (stem cell inhibition, SCI) specifically inhibit the proliferation of CFU-S both in vitro and in vivo (155, 156) and has recently been characterized as macrophage inflammatory protein-1 alpha (MIP-1 alpha) (142). Another inhibitor, a tetrapeptide characterized by Frindel & Guigon (157), exerts a similar effect. Thus, at least three factors have now been characterized that can influence the proliferation of multipotent cells and two of these molecules, TGF-beta and the tetrapeptide, also influence proliferation of more developmentally-restricted progenitor cells (153, 157–159). This diversity of growth inhibitory molecules is reminiscent of the hematopoietic growth factors with their overlapping target cell popula-

tions; however, the apparent redundancy may be misleading since the full biological spectra of the growth inhibitory molecules have not yet been determined and, in any case, the stimulus for production of the various factors may well depend upon specific circumstances. Certainly, data suggest that exposure to TGF-beta for several days is required to elicit a maximal anti-proliferative effect while maximal growth inhibition in response to MIP-1 alpha (SCI) occurs within a few hours. What are the potential uses of these growth inhibitory molecules? One exciting possibility could be to inhibit the proliferation of stem cells and their progeny prior to treatment with cell-cycle specific cytotoxic agents. The hematopoietic growth factors could then be used after chemotherapy to enhance regeneration of the cells. In patients with malignancy, of course, such a protocol demands that the corresponding tumor cells are refractory to the growth inhibitors. At least with most leukemic cells, this appears to be the case. A variety of lymphoid and myeloid leukemic cell lines have been shown not to be growth inhibited in the presence of TGF-beta (154, 160). Similarly, at least one of the defects in the putative stem cells of chronic myeloid leukemia lies in their ability to evade normal growth inhibitory factors present in long-term bone marrow cultures (161). Clearly, further studies are needed on stem cells of other normal tissues and of solid tumors. Nonetheless, the data available are encouraging and suggest not only a use of growth inhibitory molecules for the management of patients with malignant diseases, but also a possible approach at the mechanistic level to determine how tumor cells evade normal growth regulatory mechanisms.

In the longer term, knowledge of the receptors for the various cytokines, the possibility of manufacturing agonists/antagonists, an understanding of the signals transduced by the growth stimulatory and inhibitory molecules, and the molecular processes set in motion may yield information that could be employed in patients with a variety of disorders. Also, most biologists are convinced that yet more growth regulatory molecules will be found.

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