Human Macrophage Colony-Stimulating Factor Levels Are Elevated in Pregnancy and in Immune Thrombocytopenia

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Plasma macrophage colony-stimulating factor (M-CSF) levels were measured by enzyme immunosorbent assay (ELISA) using horse and rabbit polyclonal antibodies raised against human M-CSF purified from urine (hM-CSF). Plasma M-CSF levels in nonpregnant female controls were 364 ± 69 U/mL (mean ± SD, n = 20). Pregnancy results in significant elevation of circulating M-CSF levels (541 ± 164 U/mL, n = 46, P < .0005). M-CSF levels were increased by 28 weeks' gestation and did not increase further in later pregnancy. M-CSF levels were also measured in 20 female controls before and after commencing on the oral contraceptive pill. There was no effect of the contraceptive pill on plasma M-CSF levels (364 ± 69 U/mL before v 373 ± 66 U/mL after commencing the pill). In 28 nonpregnant patients with untreated immune thrombocytopenic purpura (ITP), plasma M-CSF levels were significantly increased (797 ± 402 U/mL, n = 28, v 364 ± 69 U/mL in controls, N = 20, P < .0005). Pregnant ITP patients had higher levels of plasma M-CSF (929 ± 327 U/mL, n = 25) than nonpregnant patients, but this difference was not significant. Elevated levels of M-CSF in ITP may reflect activation of the reticuloendothelial system (RES), which could result in positive feedback to increase the destruction of platelets. The increase in M-CSF associated with pregnancy could contribute to the exacerbation of latent ITP in pregnancy.

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ACROPHAGE colony-stimulating factor (M-CSF/CSF-1) is a myeloid growth factor that stimulates the proliferation and differentiation of mononuclear phagocytic progenitors. It also enhances the survival of mature circulating monocytes and tissue macrophages,2 and augments the effector functions of these cells,3 including antibody-dependent cytotoxicity,4 tumor cell cytolysis,5 phagocytosis and microbial killing,6 chemotaxis,7 and respiratory burst activity.8 Exposure of monocytes/macrophages to M-CSF induces expression of maturation-linked surface antigens such as HLA-DR, the leukocyte integrins CD11b and CD11c, and Fe-receptors,9 production of tissue plasminogen activator (t-PA),10 thromboplastin,11 and prostaglandin E1,12 and also the synthesis and secretion of cytokines including interleukin-1 (IL-1),13 granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor (TNF), and interferon gamma (IFN-γ).13,14 M-CSF has also been shown to play a role in placentation and function.15

M-CSF is produced by fibroblasts and bone marrow stromal cells in culture.3 These cells are probably responsible for the levels of M-CSF detected in the serum of normal animals and humans. Monocytes and macrophages can be stimulated to synthesize and secrete the growth factor following adherence to solid substrates,16 exposure to phorbol esters17 and cytokines such as IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α.18-20 T cells and endothelial cells can also be stimulated to produce M-CSF.21,22 In addition, uterine glandular epithelial cells have been shown to produce M-CSF in response to estrogen and progesterone in vivo,15 and, in the mouse, pregnancy results in an elevation of serum and tissue M-CSF levels.23 In humans, CSF-1 is present in the amniotic fluid of pregnant women, and levels increase twofold from 16 to 40 weeks of gestation.24

Hence, autocrine/paracrine stimulation of M-CSF release by various cell types serves to enhance the immune responses of mononuclear phagocytes. Such paracrine/autocrine loops may be of significance in clinical conditions in which there is chronic activation of the reticuloendothelial system (RES). Immune thrombocytopenic purpura (ITP) is a clinical disorder characterized by a shortened platelet survival as a result of reticuloendothelial (RE) sequestration.25-27 Accelerated platelet destruction is mediated mainly by IgG antibodies directed against platelet surface antigens.28-31 Recognition and binding of IgG-coated platelets by macrophage Fe-receptors32 leads to sequestration of opsonized platelets in the RES. Additionally, binding of IgM antibodies to platelet surface antigens33 results in complement activation34 and platelet destruction, either directly or via the macrophage complement receptor.35

In this study, we set out to determine whether pregnancy elevates circulating levels of M-CSF in humans. We also measured the levels in normal women before and after commencing on the oral contraceptive pill, to assess the effect of exogenously administered ovarian hormones on human M-CSF levels. Finally, we measured plasma M-CSF concentrations in patients with ITP to determine if they were elevated in this condition, and to investigate the effect of pregnancy on the levels of this growth factor in ITP.

MATERIALS AND METHODS

Patient characteristics and sample processing. Citrated venous blood (1 vol of 0.13 mol/L trisodium citrate to 9 vol of blood) was taken from nonpregnant female controls, before and after commencing on the combined estrogen/progestogen oral contraceptive pill, from normal antenatal patients at various gestation periods, and from pregnant and nonpregnant patients with ITP. Four types of contraceptive pill were used; all are licensed monophasic
were between the ages of 18 and 40 years, and Table 1 shows the age range and median ages of each of the patient groups studied. There were no significant differences in the ages among the groups studied. Patients with ITP were not on treatment when tested; they were either seen at first presentation, or were refractory to treatment. Cord blood was obtained at time of delivery of term infants by umbilical vein sampling. Plasma was prepared by centrifugation of citrated samples at 2,000 × g for 20 minutes, and aliquots were frozen at −70°C until use.

**M-CSF enzyme-linked immunosorbent assay.** Plasma M-CSF concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) employing antibodies raised human M-CSF purified from urine (hM-CSF), which has a specific activity of 1.5 × 10^8 U/mg (1 U being defined as the amount of hM-CSF needed to form a colony as determined by a mouse colony-forming assay). Antibodies were prepared and purified as previously described, and were kindly provided by Alpha Therapeutics, Osaka, Japan. Briefly, 96-well, flat-bottomed polystyrene plates (Costar 3590; High Wycombe, UK) were coated with 100 μL of coating solution (consisting of horse anti-hM-CSF immunoglobulin fraction, diluted in phosphate-buffered saline [PBS] containing 0.02% sodium azide to an absorbance at 280 nm of 0.1) for 24 hours at 4°C. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS/Tween), and wells were filled with 300 μL of blocking solution (10% immobilized normal horse serum, 0.25% bovine serum albumin [BSA], 0.02% sodium azide in PBS) to block nonspecific binding of other proteins. The coated plate filled with blocking solution was sealed and stored at 4°C until use (within 1 month).

Before use, contents of each plate were discarded, and the plates were washed three times with PBS/Tween. Test samples and hM-CSF standards, diluted 1 in 10 with dilution buffer (0.25% BSA, 0.3% gelatin, 0.02% sodium azide in PBS), were added to wells in duplicate, and incubated overnight at 4°C. Following three washes with PBS/Tween, rabbit polyvalent anti-hM-CSF antibody (100 μL/well) was added, and incubation continued for 2 hours at 37°C. The concentration of the rabbit anti-hM-CSF immunoglobulin used in this step was adjusted by diluting with blocking solution so that the rabbit protein had an absorbance at 280 nm of 0.5. After another three washes, horse radish peroxidase-conjugated goat IgG against rabbit IgG (0.005 to 0.05 μg/mL, Alpha Therapeutics) was added (100 μL/well) and left for 2 hours at room temperature. The wells were washed five times before addition of 100 μL of substrate (o-phenylene diamine [OPD], 0.8 mg/mL, in 0.1 mol/L citrate buffer, pH 5, with 0.015% H₂O₂) to each well. After 30 minutes in the dark at room temperature, the reaction was stopped with 2N H₂SO₄. The color was read on an automatic plate reader (Dynatech Laboratories,Billinghurst, UK), using dual-beam wavelengths of 490 nm and 690 nm. The concentration of M-CSF was calculated from the corresponding standard curve.

The linear region of the standard curve was adjusted by varying the concentration of the conjugated antibody, thus giving a range of this assay from 10 to 2,000 U/mL, ie, the lower limit of detection was 100 U/mL or 660 pg/mL of M-CSF in a plasma sample. The interassay variability was 12% and intraassay variability was 5%.

All antibodies were obtained from Alpha Therapeutics, Green Cross Corporation, Osaka, Japan.

**Platelet-associated immunoglobulins.** An ELISA was used to quantitate platelet-associated IgG (PAIgG) and IgM (PAIgM) in patients with ITP.

**Statistics.** Patient groups were compared using both the Student's t-test and the Mann-Whitney U test for nonparametric data. Where significance values are given, the test used is specified, unless the level of significance achieved is identical for both tests.

**RESULTS**

**Levels of M-CSF in normal female controls.** Plasma M-CSF levels in 20 nonpregnant female controls were 364 ± 69 U/mL (mean ± SD), with a range of 220 to 475 U/mL.

**Circulating M-CSF concentrations are increased in pregnancy.** Pregnancy results in significant elevation of plasma M-CSF levels, as measured in 46 normal healthy pregnant women who had circulating M-CSF concentrations of 541 ± 164 U/mL, with a range of 210 to 940 U/mL, P < .0005 (Fig 1). M-CSF levels were increased by 28 weeks of gestation and did not increase further in later pregnancy. Subjects who were less than 28 weeks pregnant had circulating M-CSF levels of 519 ± 199 U/mL (range, 210 to 940 U/mL, n = 19), as compared with subjects at 28 or more weeks of gestation, who had levels of 557 ± 132 U/mL (range, 360 to 820 U/mL, n = 27, NS by Students' t-test, and by Mann-Whitney U test). This was further confirmed in two subjects in whom serial samples were obtained between 20 and 38 weeks of gestation. Figure 2 shows that, in these two women, there was no consistent change in plasma M-CSF concentration with the length of gestation. In two other

| Table 1. Age Characteristics of Subject Groups Studied |
|---------------------------------|--------------|--------------|--------------|--------------|
| Nonpregnant | Pregnant | Nonpregnant | Pregnant |
| Controls | Controls | ITP Patients | ITP Patients |
| No. of subjects | 20 | 27 | 28 | 30 |
| Median age (yr) | 29 | 27 | 28 | 30 |
| Age range (yr) | 18–35 | 17–37 | 19–40 | 18–38 |

![Fig 1. M-CSF levels in normal nonpregnant and pregnant women.](https://www.bloodjournal.org)
ELEVATED HUMAN M-CSF LEVELS

Fig 2. Effect of gestation length on M-CSF levels in pregnancy. Plasma M-CSF levels were measured in serial samples taken throughout pregnancy in two healthy subjects.

Fig 3. Effect of the combined oral contraceptive pill on plasma M-CSF levels in normal women. Plasma samples were obtained from 20 women before and after commencing on the pill. M-CSF levels were determined as described.

Fig 4. M-CSF levels in ITP. Plasma samples from patients with ITP (untreated or refractory) were assayed for M-CSF concentrations as described. Data are shown with medians, means ± SDs.

subjects, M-CSF levels demonstrated a decrease from 844 U/mL and 550 U/mL (measured in the third trimester) to 400 U/mL and 270 U/mL, respectively, at 2 weeks after delivery.

M-CSF levels are not altered by the contraceptive pill. The elevated circulating levels of M-CSF in pregnancy may simply reflect the increased estrogen and/or progesterone levels, and so plasma samples from 20 female controls, taken before and 3 months after commencing on the combined oral contraceptive pill, were also assayed for M-CSF. There was no significant effect of the oral contraceptive pill on plasma M-CSF concentrations (Fig 3; NS by paired t-test). Furthermore, when analyzed separately, there was no difference among the four different preparations of the pill in their effect on plasma M-CSF levels.

M-CSF levels are elevated in umbilical cord blood of healthy term infants. High circulating levels of M-CSF in pregnancy may derive from uterine/placental sources of this cytokine. Hence, we measured M-CSF concentrations in the cord blood of 10 healthy term infants at delivery. M-CSF levels in cord blood were 946 ± 190 U/mL (range, 560 to 1,180 U/mL, n = 10), which is significantly higher than those found in healthy nonpregnant or pregnant controls (364 ± 69 U/mL and 540 ± 164 U/mL, respectively, P < .0005 for both).

M-CSF levels are increased in ITP. Plasma levels of M-CSF are significantly increased in nonpregnant female patients with ITP (797 ± 402 U/mL, with a range from 335 to 2,050 U/mL, n = 28; v 364 ± 69 U/mL in nonpregnant controls, n = 20, P < .0005; Fig 4). A separate group of patients with ITP in pregnancy had higher levels of plasma M-CSF when compared with the group of nonpregnant ITP patients, but this difference did not reach significance (929 ± 327 U/mL, range 560 to 1,800 U/mL, n = 25 in pregnant patients, v 797 ± 402 U/mL, range 335 to 2,050 U/mL, n = 28, in nonpregnant patients, 1 < P < .375 by Student's t test). M-CSF levels in pregnant ITP patients were significantly higher than in pregnant controls (929 ± 327 U/mL, n = 25, v 541 ± 164 U/mL, n = 46, P < .0001).

There was no correlation between M-CSF levels and either PAIgG or PAIgM in pregnant or nonpregnant patients with ITP (data not shown).
DISCUSSION

In this study, plasma M-CSF levels in normal nonpregnant women are 364 ± 69 U/mL (mean ± SD), with a range of 220 to 475 U/mL. These levels are two to three times higher than has been reported by investigators using a radioimmunoassay (RIA), and rabbit antibody raised against recombinant M-CSF (rM-CSF)38 (118 ± 9 U/mL), but agree much more closely with the results obtained by another study (540 ± 110 U/mL)39 which used the same ELISA as we have in this study. This discrepancy may reflect the difference in specific activities between the recombinant product and hM-CSF. Shadle et al reported a specific activity of their rhM-CSF of 5.19 × 10⁷ U/mg, while hM-CSF used as a standard in our assays has a specific activity of 1.5 × 10⁶ U/mg.36 Another study that used a RIA based on a rhCSF-1 with a specific activity of 8.3 × 10⁷ U/mg reported serum CSF-1 concentrations in normal subjects to be 372 ± 111 U/mL, with a range of 144 to 700 U/mL,39 which is in close agreement with our data. On the other hand, Shadduck et al, using a RIA based on purified human urinary CSF-1 with a specific activity of 2 × 10⁷ U/mg, reported M-CSF values in 10 normal volunteers to be 113 ± 21 U/mL.40 Finally, in a RIA based on a CSF-1 with a specific activity of 4.5 × 10⁶ U/mg, M-CSF levels in normal controls were measured at 174 ± 76 U/mL.41 On the basis of these different activities, the serum or plasma hM-CSF levels reported by all these studies are calculated to fall within the range of 2.3 to 4.0 ng/mL, and are therefore in close agreement.

We have demonstrated that circulating levels of M-CSF are significantly elevated in pregnancy. Similarly, Hanamura et al36 found that serum M-CSF levels in 10 pregnant women were higher than in normal controls, and Ringler et al42 reported a modest elevation in serum CSF-1 levels in pregnant women. The elevated levels of M-CSF seen in pregnancy may be due to the stimulation of uterine glandular epithelial cells by ovarian hormones (estradiol 17b and progesterone).15 We have also measured the concentrations of M-CSF in the cord blood of full-term infants at delivery, and found these to be significantly higher than the levels in nonpregnant adult women, and also significantly higher than maternal levels measured throughout pregnancy in normal women. This may relate to the hormonal changes associated with labor and delivery, and reflect a surge in M-CSF levels at the time. We have not been able to demonstrate any effect of the combined oral contraceptive pill on M-CSF levels. However, it should be noted that in pregnancy the actual numbers of uterine epithelial cells are greatly increased.

We have also demonstrated that circulating M-CSF levels in female patients with ITP are significantly increased when compared with age-matched female controls. High circulating levels of M-CSF in ITP may reflect the degree of macrophage activation in this condition. Similarly, elevated levels of CSF-1 in patients with myeloproliferative disorders may reflect increased production of this growth factor by activated monocytes and macrophages.42 We did not find any correlation between circulating M-CSF levels and the levels of platelet-associated immunoglobulins (PAIgG and PAIgM) in the group of patients studied here. This is not entirely surprising, as total platelet immunoglobulin is increased in thrombocytopenia as a result of increased platelet destruction, whether due to immune or nonimmune causes, and does not necessarily reflect the degree of activation of the RES.39

The exact role of the growth factor in the pathophysiology of the disease is unclear. Monocyte-platelet interactions have been demonstrated in ITP, and correlate well with the amount of platelet surface-bound IgG (PIgG).28 Such receptor-mediated interactions, resulting in macrophage activation, and the subsequent synthesis and release of M-CSF, either by macrophages themselves or by other cells stimulated to produce M-CSF by monokines, could lead to autocrine stimulation, thus increasing the destruction of antibody-coated platelets. M-CSF has been reported to enhance the expression of both FcRI and FcRII on mature macrophages.43 Additionally, low platelet counts in ITP could be accompanied by secretion of other growth factors such as interleukin-6 (IL-6)44 by unknown feedback mechanisms. These growth factors could in turn stimulate M-CSF production by mononuclear cells, endothelial cells, or T cells. Such paracrine/autocrine loops may also be responsible for the increased levels of CSF-1 that have been reported in lymphoid, as well as myeloid, malignancies.39

Evidence for a biological effect of increased M-CSF levels is seen in pregnancy. In pregnant mice, elevated tissue and serum levels of M-CSF correlate with increased monocytopoiesis, as evidenced by a fivefold increase in the concentration of circulating monocytes, and a greater than twofold increase in the number of splenic macrophage precursor cells (CFU-C).21 In hematologically normal patients with malignant lymphoma, clinical administration of hM-CSF at doses ranging from 4 to 16 mU/m², resulting in peak serum levels of 1,000 to 2,000 U/mL, led to an enhancement of mononuclear phagocyte functions, including migration into skin windows, priming of the respiratory burst, and phagocytosis/killing of Candida.45 These patients also demonstrated small but significant reductions in platelet counts by the end of a 2-hour infusion of hM-CSF, which may have been secondary to increased phagocytic clearance of platelets. Hence, elevated levels of M-CSF may play an important part in the persistent destruction of platelets in some cases of chronic ITP.

It is possible that the increased production of M-CSF might contribute to the exacerbations of ITP seen in pregnancy. In this study, patients with ITP in pregnancy did have higher levels of plasma M-CSF than nonpregnant patients with ITP, but this difference was not significant. Monocytes are able to specifically regulate circulating levels of M-CSF by receptor-mediated endocytosis and intracellular degradation.46 The ability of mononuclear phagocytes to degrade receptor-internalized M-CSF increases by at least 10-fold during differentiation, thus providing a mechanism for controlling the production of mature mononuclear phagocytes. Hence, it may be that in patients with ongoing...
activation of the RES producing large amounts of M-CSF, this homeostatic mechanism may override the increased production of the growth factor by uterine cells in pregnancy. On the other hand, the increased production of M-CSF by epithelial cells in the pregnant uterus, resulting in macrophage activation and enhanced expression of Fc-receptors, could lead to exacerbations of ITP in pregnancy.

In conclusion, circulating M-CSF levels are significantly elevated in pregnancy, and in patients with untreated ITP. The high levels found in ITP may reflect the activation of mononuclear phagocytes in this condition, and could, in turn, result in positive feedback to increase the destruction of platelets. Although patients with ITP in pregnancy do not appear to have higher circulating levels of M-CSF than nonpregnant patients with ITP, it may be that increased production of M-CSF by uterine epithelial cells may contribute to the exacerbation/uncovering of latent ITP in pregnancy.

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