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Transcriptional Rates of Granulocyte-Macrophage Colony-Stimulating Factor, Granulocyte Colony-Stimulating Factor, Interleukin-3, and Macrophage Colony-Stimulating Factor Genes in Activated Cord *Versus* Adult Mononuclear Cells: Alteration in Cytokine Expression May Be Secondary to Posttranscriptional Instability[1]

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ABSTRACT. We have previously demonstrated that protein production and mRNA expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and IL-3 are decreased in activated mononuclear cells (MNC) from human umbilical cord compared with adult peripheral blood. Reduced production of these colony-stimulating factors (CSF) during states of increased demand, as occurs during overwhelming bacterial infection, may play a role in the pathogenesis of neutropenia and thrombocytopenia in the newborn. To determine whether the reduced mRNA expression and CSF production from activated cord MNC is secondary to the decreased transcriptional activity of the corresponding genes, we determined the transcriptional rate of GM-CSF, G-CSF, IL-3, and M-CSF by nuclear run-on assays. Cord and adult MNC were isolated by Ficoll-Hypaque density centrifugation. A total of 10^8 MNC from cord and adult blood were stimulated as follows: GM-CSF and G-CSF [32 nmol/L phorbol-12-myristate-6-acetate (20 μg/L) + 2 mg/L phytohemagglutinin for 6 h]; IL-3 [32 nmol/L phorbol-12-myristate-6-acetate (20 μg/L) + 0.5 μmol/L A 23187 for 6 h]; and macrophage CSF (2 μg/L recombinant human GM-CSF for 24 h). The nuclei from unstimulated and stimulated cells were isolated and labeled with ^{32}P-uridine triphosphate. Newly elongated ^{32}P-labeled RNA transcripts were hybridized to slot blots of CSF DNA. To minimize cross hybridization artifacts, short fragments (0.5–1.0 kb) of cDNA were used. The transcriptional rate increase of GM-CSF, G-CSF, IL-3, and macrophage CSF upon stimulation appears to be similar in both cord and adult MNC [GM-CSF: 260 ± 62% (cord) *versus* 270 ± 33% (adult); G-CSF: 220 ± 71% (cord) *versus* 220 ± 44% (adult); IL-3: 150 ± 25% (cord) *versus* 160 ± 38% (adult); macrophage CSF: 130 ± 10% (cord) *versus* 150 ± 15% (adult), mean ± SD]. These findings indicate that cord MNC transcribe these CSF genes at the same level as adult MNC during states of increased demand (stimulation). Therefore, the decrease in CSF mRNA expression in activated cord *versus* adult MNC is probably not secondary to defects in transcriptional regulation. Alteration in posttranscriptional events, such as dysregulation of mRNA stability, could account for the difference between newborn and adult CSF expression. (*Pediatr Res* 34: 560–564, 1993)

Abbreviations

CSF, colony-stimulating factor
GM-CSF, granulocyte-macrophage colony-stimulating factor
G-CSF, granulocyte colony-stimulating factor
M-CSF, macrophage colony-stimulating factor
MNC, mononuclear cell
PMA, phorbol-12-myristate-6-acetate
PHA, phytohemagglutinin
A, adenine
U, uracil
SSC, standard saline citrate

The regulation of hematopoiesis is developmentally immature in the newborn compared with the adult (1). These deficiencies may contribute to the high incidence of neonatal cytopenias during states of increased demand, such as bacterial sepsis (1, 2). The immaturity of host defense in neonates appears to be the result of multiple factors, including reduced myeloid effector cells, early myeloid progenitor cells (3, 4), and delayed induction of hematopoietic progenitor cell cycling (5).

Proliferation and differentiation of hematopoietic progenitor cells and the regulation of hematopoiesis are controlled by highly specific hematopoietic growth factors (CSF and IL) (6–9). GM-CSF plays a major role in myelopoiesis by stimulating the proliferation and differentiation of hematopoietic progenitor cells from granulocytic and monocytic lineages (10, 11). Effector functions of mature cells, such as neutrophils, eosinophils, macrophages, and endothelial cells, are also enhanced by GM-CSF

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(12–15). G-CSF stimulates myeloid progenitor proliferation, induces egress of mature neutrophil storage pool cells from the bone marrow into the peripheral blood, and enhances mature neutrophil effector function (16–18). IL-3 supports the proliferation and differentiation of a range of hematopoietic progenitor cells *in vitro*, giving rise to granulocytic, monocytic, erythroid, mast cell, megakaryocytic, and mixed colonies (8, 19). In addition, human IL-3 stimulates mature basophils, eosinophils, and monocytes to become functionally activated (8, 20, 21). IL-3 is distinct among the latter CSF in possessing the capacity to stimulate progenitor cell renewal (22). Macrophage-specific CSF (M-CSF, also referred to as CSF-1) stimulates hematopoietic stem cells to form colonies containing predominantly monocytes and macrophages (23, 24).

Recently, we have shown that protein production and mRNA expression of GM-CSF, G-CSF, and IL-3 are decreased in activated MNC from human cord blood compared with adult peripheral blood (25, 26). Our previous studies have also demonstrated that the affinity, binding, and number of GM-CSF and G-CSF receptors on cord peripheral mature effector cells are similar to adult cells (25, 26). Reduced mRNA expression and protein production of tumor necrosis factor-α and γ-interferon from activated human neonatal MNC *versus* adult MNC have also been described by others (27–30). Reduced production of these CSF during states of increased demand such as overwhelming bacterial sepsis may play a role in the pathogenesis of peripheral neutropenia and thrombocytopenia.

In the present study, we attempted to elucidate the mechanisms of decreased CSF mRNA expression and production from activated cord *versus* adult MNC to understand the underlying mechanisms associated with the interrelationships between neonatal CSF gene dysregulation and the immaturity in neonatal host defense. We hypothesized that a decreased transcriptional rate of GM-CSF, G-CSF, and IL-3 from newborn MNC could account for the previously described decrease in CSF mRNA expression from newborn activated MNC. Therefore, we determined the transcriptional rate of GM-CSF, G-CSF, IL-3, and M-CSF by nuclear run-on assays. In this study, we also used shorter size fragments of CSF cDNA spanning most of the coding regions but eliminating the sequence elements that are repeated in the genome, for example, poly (A) tract. These latter sequences could cross hybridize within the heterogeneous population of radiolabeled RNA generated by the run-on reactions (31). Our results suggest that the decreased production of GM-CSF, G-CSF, and IL-3 and reduced mRNA expression from activated neonatal MNC *versus* adult MNC are not secondary to defects in transcriptional regulation.

MATERIALS AND METHODS

MNC isolation and culture condition. Peripheral blood was obtained by venipuncture from healthy adult volunteers in accordance with the principles of the Declaration of Helsinki. Blood samples were also obtained from the umbilical cord of the placenta of normal, full-term, nonstressed infants immediately after scheduled cesarean section. The samples were collected in heparinized syringes. Cord and adult MNC were isolated from whole blood by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient density centrifugation (density = 1.007 g/mL) at 400 × *g* for 30 min at room temperature. The MNC at the interface were collected, washed twice with Dulbecco's PBS (Sigma) and resuspended in RPMI-1640 (Gibco, Grand Island, NY) culture medium supplemented with antibiotics and 10% heat inactivated human AB serum (Sigma). MNC isolated by this density gradient separation were purified to greater than 98% homogeneity. There was no difference in the MNC differential between cord and adult (adult: 86 ± 4.0% lymphocytes and 7.2 ± 3.0% monocytes, cord: 82 ± 8.0% lymphocytes and 8.8 ± 4.0% monocytes). The cells were plated at a density of 1×10^6 cells/mL in culture medium and stimulated with specific stimuli.

Nuclear run-on transcription assay. A total of 10^8 MNC from cord and adult blood of three different samples were stimulated as follows to get optimal expression of each cytokine mRNA as described previously (25, 26): for GM-CSF and G-CSF studies, 32 nmol/L PMA (20 μg/L) + 2 mg/L PHA for 6 h; for IL-3, 32 nmol/L PMA (20 μg/L) + 0.5 μmol/L A23187 for 6 h; and for M-CSF, 2 μg/L rhGM-CSF for 24 h. Nuclei isolation and nuclear run-ons were done using slight modification of a previously described technique (32). The cells were washed in PBS, lysed in 4 mL of lysis buffer (10 mmol/L Tris, pH 7.4, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.5% NP-40) on ice for 5 min, and centrifuged for 5 min at 1500 rpm (4°C) in a Beckman TJ-6 centrifuge to pellet the nuclei. The nuclei were washed in lysis buffer, then resuspended in 100 μL of storage buffer (40% glycerol, 50 mmol/L Tris, pH 7.4, 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA) and stored at −70°C until use. Nuclear run-on reactions were carried out for 30 min at 30°C in 50 μL of elongation buffer (20 mmol/L Tris, pH 8.0, 10 mmol/L $MgCl_2$, 100 mmol/L KCl, 10 mmol/L DTT) after the addition of 5 μL each of 10 mmol/L ATP, guanidine triphosphate, and cytidine triphosphate, 200 μCi of [^{32}P]-uridine triphosphate (3000 Ci/mmol, Amersham, Arlington Heights, IL), 5 U of RNAsin (Promega, Madison, WI), and 20 μL of H_2O. The reaction was stopped by the addition of 40 IU of RNase-free DNase and further incubated at 30°C for 10 min. Ten μL of stop buffer [10% Sarcosyl (International Biotechnologies, Inc., New Haven, CT), 100 mmol/L EDTA, 100 mmol/L Tris, pH 7.6, 1 g/L proteinase K] were added and heated at 42°C for 30 min. Fifteen μg of yeast transfer RNA (17 U/mg, Sigma) were added as carrier. The RNA was extracted three times with phenol/chloroform/isoamylalcohol (25:24:1, vol/vol) and precipitated in ethanol using 0.3 mol/L sodium acetate (pH 4). Newly elongated ^{32}P-labeled RNA transcripts were hybridized to membrane bound DNA probes.

Target DNA samples used in the hybridizations include the following: GM-CSF, 800-bp *Xho*I fragment, 550-bp *Xho*I/*Nco*I fragment, and 250-bp *Nco*I/*Xho*I fragment from pGM-CSF (Genetics Institute, Cambridge, MA); G-CSF, 1800-bp *Xho*I fragment, 800-bp *Xho*I/*Stu*I fragment, and 700 bp-*Stu*I/*Stu*I fragment from pG-CSF 2.2 (Genetics Institute); IL-3, 1000-bp *Xho*I fragment, 600-bp *Eco*RI/*Xho*I fragment, and 400-bp *Xho*I/*Eco*RI fragment from pHucIL3-2 (American Type Culture Collection, Rockville, MD); M-CSF, 1800-bp *Eco*RI/*Xho*I fragment, 700-bp *Bam*HI/*Bam*HI fragment, and 880-bp *Eco*RI/*Bam*HI fragment from p3ACSFRI (Genetics Institute) (Fig. 1). DNA samples were denatured by adding NaOH to a final concentration of 0.1 mol/L NaOH and incubating at room temperature for 30 min. Four volumes of 6 × SSC were then added and same amount of DNA solution was loaded onto nitrocellulose (pore size 0.2 μM, Schleicher & Schuell, Keene, NH) using a minifold II slot blot apparatus (Schleicher & Schuell). The amount of full-length cDNA per slot was 1 μg. The amount of short-size fragment DNA was adjusted to be the same as cDNA in molar concentration according to their base pair numbers. PUC 19 was included as a negative control as the plasmid vector. β-Actin was also included as an internal control for normalization.

Before the hybridization, the filters were baked for 2 h at 80°C and were prehybridized for at least 1 h in hybridization solution (50% formamide, 5 × SSC, 1 × Denhardt's, 50 mmol/L sodium phosphate, pH 6.5, 0.1% SDS, and 50 mg/L denatured single-strand sperm DNA). Hybridizations were carried out using 5 to 10 million cpm/mL of hybridization solution at 42°C for 36 h. After hybridization, the slot blots were washed to 0.3 × SSC at 65°C for 1 h, then exposed to Kodak XAR-5 x-ray film with intensifying screen at −70°C for 2–7 d. Run-on signal strengths were determined by densitometry of autoradiographs. The density of the bands was calculated by normalizing values with respect to the signals of internal standards.

Statistical analysis. Results are expressed as mean ± SD of three samples. The probability of significant differences when

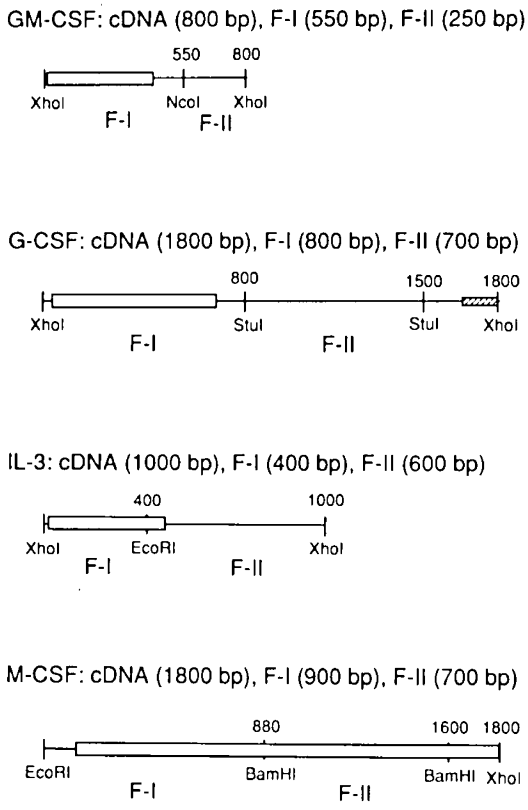

Fig. 1. Restriction maps of target DNA. Target DNA samples used in the hybridizations included the following: GM-CSF: cDNA = 800-bp *Xho*I fragment, F-I = 550-bp *Xho*I/*Nco*I fragment, F-II = 250 bp *Nco*I/*Xho*I fragment from pGM-CSF; G-CSF: cDNA = 1800-bp *Xho*I fragment, F-I = 800-bp *Xho*I/*Stu*I fragment, F-II = 700-bp *Stu*I/*Stu*I fragment from pG-CSF 2.2; IL-3: cDNA = 1000-bp *Xho*I fragment, F-I = 400-bp *Xho*I/*Eco*RI fragment, F-II = 600-bp *Eco*RI/*Xho*I fragment from pHucIL3-2; and M-CSF: cDNA = 1800-bp *Eco*RI/*Xho*I fragment, F-I = 880-bp *Eco*RI/*Bam*HI fragment, F-II = 700-bp *Bam*HI/*Bam*HI fragment from p3ACSFRI. The *open box* represents the translated region for mature protein. The *shaded box* represents poly (A) sequences.

comparing two groups was determined with the use of the unpaired *t* test. p values < 0.05 were considered significant.

RESULTS

Nuclear run-on transcriptional assays for GM-CSF, G-CSF, IL-3, and M-CSF genes were performed to determine whether the reduced cytokine production and mRNA expression in activated cord MNC was mediated at the level of gene transcription. The nuclei from unstimulated and stimulated cells of three different samples were isolated and incubated with [^{32}P]-uridine triphosphate. Newly elongated ^{32}P-labeled RNA transcripts were hybridized to membrane-bound target DNA. No hybridization signal was observed on the slot blots containing control DNA. The transcriptional activity of each CSF gene was measured by quantitative densitometry, normalized with β-actin intensity, and expressed as percent increase of densitometric values compared with the values of unstimulated cells.

Transcriptional rate of GM-CSF gene in activated cord and adult MNC. As shown in Figure 2, unstimulated MNC from both cord and adult showed negligible basal level signals of the GM-CSF gene. Six h after PMA and PHA stimulation, the transcriptional rate of the GM-CSF gene was increased approximately 3-fold in both cord and adult MNC (Fig. 2). However, there was no appreciable differences between activated cord and adult MNC in the degree of transcriptional activation [260 ± 62% (cord) *versus* 270 ± 33% (adult), $n = 3$] (Table 1).

Transcriptional rate of G-CSF gene in activated cord and adult MNC. As shown by a representative autoradiogram of G-CSF gene transcription assay (Fig. 3), the basal level signal of this gene was apparent with cDNA as a target in both cord and adult MNC. However, the basal level signal measured by fragment I or fragment II as targets was only 25% of the signal seen using cDNA. The basal level signal of this gene was approximately the same between cord and adult MNC. Stimulation of cells for 6 h with PMA and PHA caused approximately 2-fold increase in both cord and adult MNC as compared with the G-CSF transcription rate in unstimulated cells. As in the GM-CSF case, there was no appreciable difference between activated cord and adult MNC in the extent of transcriptional activation [220 ± 71% (cord) *versus* 220 ± 44% (adult), $n = 3$] (Table 1).

Transcriptional rate of IL-3 gene in activated cord and adult MNC. The basal level signal of the IL-3 gene was apparent with

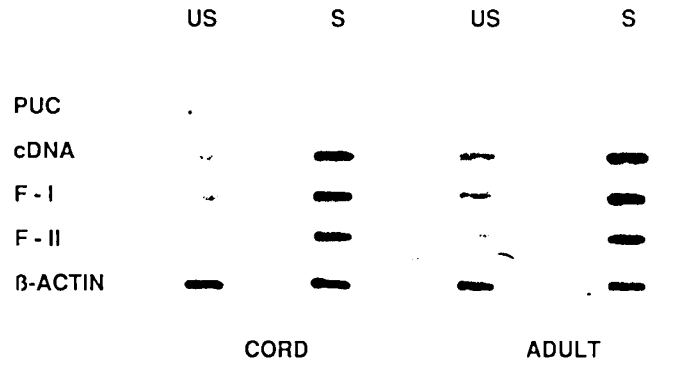

Fig. 2. Nuclear run-on analysis of GM-CSF transcription in MNC stimulated with PMA/PHA from both cord and adult peripheral blood. Equivalent amounts of radioactive labeled RNA were hybridized to filters containing the indicated DNA fragments ($n = 3$). *US*, unstimulated; *S*, stimulated; *PUC*, control. F-I, 550-bp *Xho*I/*Nco*I fragment; F-II, 250-bp *Nco*I/*Xho*I fragment from pGM-CSF.

Table 1. *Transcriptional activity of GM-CSF, G-CSF, IL-3, and M-CSF in stimulated cord MNC vs adult MNC**

	Cord (%)	Adult (%)	*n*
GM-CSF	260 ± 62	270 ± 33	3
G-CSF	220 ± 71	220 ± 44	3
IL-3	150 ± 25	160 ± 38	3
M-CSF	130 ± 10	150 ± 15	3

* The data represent results with the fragment I as a target; the transcriptional activity of the above CSF genes in the stimulated cells were expressed as percent increase of densitometric values compared with the values of unstimulated cells. Data are expressed as mean ± SD.

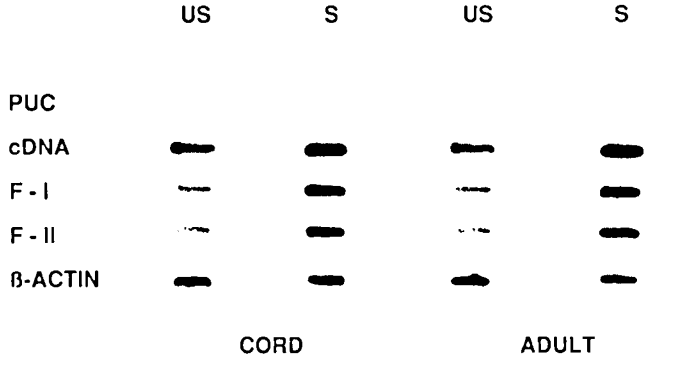

Fig. 3. Nuclear run-on analysis of G-CSF transcription in MNC stimulated with PMA/PHA from both cord and adult peripheral blood. Equivalent amounts of radioactive labeled RNA were hybridized to filters containing the indicated DNA fragments ($n = 3$). *US*, unstimulated; *S*, stimulated; *PUC*, control. F-I, 800-bp *Xho*I/*Stu*I fragment; F-II, 700-bp *Stu*I/*Stu*I fragment from pG-CSF 2.2.

cDNA or fragment II as targets, as shown in Figure 4. However, the basal level signal with fragment I was less than half of the signals (20–40%) seen with cDNA or fragment II as targets in both cord and adult MNC. The basal level signal of this gene was approximately the same between cord and adult MNC. Stimulation with PMA and A 23187 for 6 h resulted in approximately 1.5-fold increase in the IL-3 gene transcription rate in both the cord and adult MNC (Fig. 4). However, similar to GM-CSF and G-CSF transcription, the degree of transcriptional activation was virtually the same in cord and adult MNC [150 ± 25% (cord) *versus* 160 ± 38% (adult), $n = 3$] (Table 1).

Transcriptional rate of M-CSF gene in activated cord and adult MNC. As shown in Figure 5, unstimulated MNC from both cord and adult have minimal basal level signal of the M-CSF gene. Overnight stimulation with recombinant human GM-CSF induced approximately 1.5-fold increase in the M-CSF gene transcription in both cord and adult MNC. However, similar to the above results for GM-CSF, G-CSF, and IL-3 transcriptional activity, there was no appreciable difference between cord and adult MNC in the degree of transcriptional activation [130 ± 10% (cord) *versus* 150 ± 15% (adult), $n = 3$] (Table 1).

DISCUSSION

The regulation of GM-CSF, G-CSF, IL-3, and M-CSF gene expression from various type of cells from human adults has been previously examined (33–36). In contrast, the regulation of CSF gene expression in cord *versus* adult has not been extensively studied. Recently, our laboratory and others have demonstrated decreased protein production and mRNA expression of GM-CSF, G-CSF, IFN-γ, IL-6, and tumor necrosis factor-α in neonatal MNC *versus* adult (25–30). However, it was not clear whether the reduced mRNA expression of these CSF in the newborn is secondary to the decreased transcriptional activity of the corresponding genes or to alteration in posttranscriptional events.

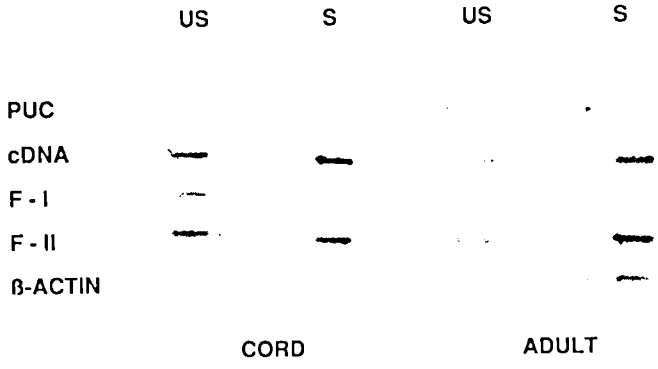

Fig. 4. Nuclear run-on analysis of IL-3 transcription in MNC stimulated with PMA/A23187 from both cord and adult peripheral blood. Equivalent amounts of radioactive labeled RNA were hybridized to filters containing the indicated DNA fragments ($n = 3$). *US*, unstimulated; *S*, stimulated; *PUC*, control. F-I, 400-bp *Xho*I/*Eco*RI fragment; F-II, 600-bp *Eco*RI/*Xho*I fragment from pHucIL-3.

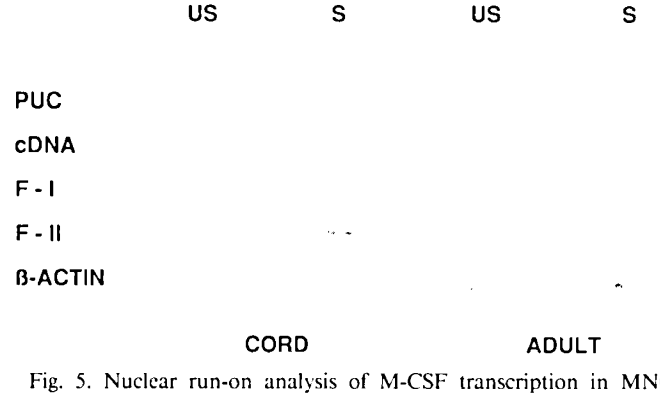

Fig. 5. Nuclear run-on analysis of M-CSF transcription in MNC stimulated with recombinant human GM-CSF from both cord and adult peripheral blood. Equivalent amounts of radioactive labeled RNA were hybridized to filters containing the indicated DNA fragments ($n = 3$). *US*, unstimulated; *S*, stimulated; *PUC*, control. F-I, 880-bp *Eco*RI/*Bam*HI fragment; F-II, 700-bp *Bam*HI/*Bam*HI fragment from p3ACSFRI.

In the present study, we determined the transcriptional rate of GM-CSF, G-CSF, IL-3, and M-CSF genes in human neonatal MNC *versus* adult. The basal level signals of each CSF gene were minimal and approximately the same between cord and adult MNC. Specifically, our nuclear run-on assays were performed with shorter size fragment DNA as target DNA to avoid the cross hybridization artifacts, as suggested by Brorson *et al.* (31). The transcriptional rate of GM-CSF, G-CSF, IL-3, and M-CSF gene after stimulation was increased significantly. These results suggest that the transcriptional activation seems to account for the enhanced gene expression of these CSF in both activated cord and adult MNC. However, the degree of transcriptional activation of the GM-CSF, G-CSF, IL-3, and M-CSF genes was virtually the same in activated cord and adult MNC (Table 1). These findings indicate that cord MNC, during states of increased demand (stimulation), transcribe these specific CSF genes at the same level as adult MNC. These results also suggest that the decreased production of GM-CSF, G-CSF, and IL-3 and reduced mRNA expression of these genes from stimulated cord MNC *versus* adult MNC are probably not secondary to defects in transcriptional regulation. Alteration in posttranscriptional regulation such as mRNA stability may, however, account for the difference between cord and adult CSF mRNA expression. Our laboratory previously reported a shorter half-life of GM-CSF mRNA in activated cord compared with adult MNC (25).

Control of mRNA stability is poorly understood, but the process is thought to involve various factors interacting with specific mRNA sequences (37, 38). The AU-rich sequences in the 3′ untranslated region of many short-living mRNA are shown to be the target of a pathway for selective processing and mRNA degradation (39–43). Certain lymphokines, cytokines, and proto-oncogenes including GM-CSF, G-CSF, IL-3, and M-CSF share these conserved AU-rich sequences in the 3′ untranslated region of their messages (39, 40, 44–47). Specifically, the 3′ untranslated regions of GM-CSF, G-CSF, and IL-3 contain repeated AUUUA motifs in a U-rich context, which are known to mediate selective mRNA degradation *in vitro* and may cause mRNA instability of the above CSF *in vivo* (39, 48). Recently, an AUUUA-specific mRNA binding protein was identified (42) and a 32-kD protein has been shown to be involved in the destabilization of mRNA through binding to AU-rich domain in the 3′ untranslated regions of rapidly degraded mRNA (49). Gillis and Malter (50) have reported another AU binding factor that recognizes the AU-rich elements of cytokine, lymphokine, and oncogene mRNA. The binding activity of this AU binding factor was induced after the activation of resting cells with agonists, resulting in subsequent mRNA stabilization (50, 51). Evidence also suggests that rapidly turned over labile proteins are involved in the mRNA degradation (52, 53). One potential target for such proteins might be the AU-rich elements in the 3′ untranslated region. It seems likely that various protein factors interacting with specific mRNA sequences (for example, AUUUA-rich motifs) exist *in vivo* and are involved in the regulation of AU-rich mRNA decay (49–51). It is also possible that any alterations in the expression and/or in the biologic activities of one or a few of these factors in cord MNC contribute to the apparent reduction of several AU-rich mRNA coding for CSF, including GM-CSF, G-CSF, and IL-3. Further experiments have to be performed to test these possibilities.

GM-CSF, G-CSF, IL-3, and M-CSF are potent regulators of hematopoietic, immune, and inflammatory systems. Deficiencies

of these CSF might severely impair the normal host defense homeostasis in the newborn. The present study has demonstrated that stimulated cord MNC transcribe GM-CSF, G-CSF, IL-3, and M-CSF genes at the same level as adult MNC. These results suggest that the decreased production of these CSF and reduced mRNA expression from stimulated cord MNC are probably not secondary to defects in transcriptional regulation.

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