Low Burst Promoting Activity Production by Phytohemagglutinin-Stimulated Mononuclear Cells of Cord Blood

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ABSTRACT. Burst-promoting activity (BPA) produced by phytohemagglutinin(PHA)-stimulated cord blood mononuclear cells (MNC) was examined using the two-stage cell culture assays. Burst-promoting activity was measured as the increase in the number of early erythroid progenitor cells in 2-day incubation of peripheral blood MNC with or without the conditioned medium of PHA-stimulated MNC (PHA-LCM). Burst-promoting activity in PHA-LCM of cord blood was significantly lower than that of adult blood (37 ± 13 versus $105 \pm 19\%$, mean $\pm$ SD, $p < 0.01$). No elevation of inhibitors to the erythroid colony growth was noted in PHA-LCM of cord blood. In contrary, the response of cord blood MNC to PHA was similar to that of adult blood MNC, as determined by colony-stimulating activity production and cell proliferation. These results showed that burst-promoting activity production by PHA-stimulated MNC of cord blood was lower than that of adult blood. (*Pediatr Res* 21: 285–288, 1987)

Abbreviations

BPA, burst-promoting activity
PHA-LCM, phytohemagglutinin-stimulated mononuclear cells conditioned medium
CSA, colony-stimulating activity
MNC, mononuclear cells
FCS, fetal calf serum
BFU-E, erythroid burst-forming unit
CFU-GM, granulocyte, monocyte colony forming unit
IMDM, Iscove's modified Dulbecco's medium
 $^3\text{H-TdR}$, tritiated-thymidine

BPA is considered to be a humoral regulator of erythropoiesis different from that of erythropoietin (1). BPA was reported to regulate the early stage of erythropoiesis whereas erythropoietin regulates the late stage of erythropoiesis. BPA was obtained from many different sources including conditioned medium of T cells (2), monocytes (3), large granular lymphocytes (4), bone marrow cells (5), and mitogen stimulated cells (6, 7).

Erythropoiesis in the early infantile period shows marked changes. The high values of hemoglobin at birth decrease rapidly and reach a nadir at about 2 months of age. Thereafter, erythropoiesis is reactivated and the hemoglobin levels return to the normal level of children. Several studies found a relationship

between the serum erythropoietin level and erythropoiesis in this period (8–10). However, the detailed mechanism of humoral regulation of erythropoiesis in this period is still unclear, and the ability of BPA production by MNC in the neonatal period has not been evaluated. In the present study, we investigated the BPA production by PHA-stimulated MNC of cord blood in comparison with that of adult peripheral blood and showed that BPA production by cord blood MNC were markedly lower than that of adult blood.

MATERIALS AND METHODS

Subjects. Cord blood was drawn from umbilical cords of normal neonates at 37–41 wk gestations with informed consent of their parents. Adult peripheral venous blood was obtained from healthy volunteers.

Preparation of conditioned medium. MNC were separated from the heparinized blood by Ficoll-Hypaque density gradient and washed twice with IMDM (GIBCO, Grand Island, NY). Unfractionated MNC were suspended at a concentration of 1×10^6 cells/ml in IMDM supplemented with 20% FCS (Irvine Scientific, Santa Ana, CA) and 1% PHA-M (Sigma, St. Louis, MO). The cells were cultured in Falcon culture flasks for 5 days at 37°C in a humidified atmosphere of 5% CO_2 in air. After 5 days of culture, the medium was aspirated from the culture flasks, centrifuged at $400 \times g$ for 10 min, passed through a $0.22 \mu\text{m}$ millipore filter, and frozen at -20°C until use for BPA and CSA assay.

BPA assay. Dukes' two-stage assay (11) was used with a little modification. MNC were separated from adult peripheral blood and adherent cells were removed as previously described (12). Nonadherent cells were suspended at a concentration of 3×10^5 cells/ml in 4 ml IMDM containing 20% FCS, 5×10^{-5} M 2-mercaptoethanol and several concentrations of PHA-LCM. As negative controls, the cells were incubated without the addition of PHA-LCM and were carried out in every experiment. After 2-day culture of the nonadherent MNC in 17×100 mm Falcon capped tissue culture tubes, the cells were collected by centrifugation at $300 \times g$ for 10 min and resuspended in 4 ml fresh medium containing 30% FCS, 5×10^{-5} M 2-mercaptoethanol, 1% bovine serum albumin (Sigma), 0.9% methylcellulose, and 1 U/ml erythropoietin (57 U/mg, Toyobo, Osaka, Japan). For the second stage of the assay, 1-ml aliquots of these cell suspensions were incubated in 35×100 mm Falcon tissue culture dishes for 10–12 days, and then evaluated for BFU-E colonies. Colonies consisting of three or more subcolonies of erythroid cells or large single accumulation of erythroid cells (>200 cells) were defined as BFU-E-derived colonies. BPA response was defined as the increases in BFU-E colonies observed in cultures containing PHA-LCM during the suspension culture stage over the numbers of colonies found in negative controls. BPA response of each

Received June 23, 1986; accepted October 27, 1986.

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sample was expressed as a percentage of the activity to standard PHA-LCM samples in our laboratories. The interassay variance in the assays was less than 15%.

CSA assay. Fresh bone marrow cells were obtained from hematologically normal patients who were undergoing routine evaluation. Nonadherent MNC were separated by the Ficoll-Hypaque procedure and plastic adherence. The nonadherent MNC were incubated at 1×10^5 cells/ml in IMDM containing 20% FCS, 0.3% agar, and PHA-LCM at several concentrations in 35×100 mm Falcon culture dishes for 9 days. CFU-GM colonies were evaluated by counting colonies containing 40 or more granulocytes-monocytes. CSA was expressed as the number of CFU-GM colonies per 1×10^5 MNC.

Assay for ^3H -TdR uptake. MNC at a concentration of 1×10^6 cells/ml were incubated in IMDM containing 20% FCS and 1% PHA-M in Falcon 96-well flat bottomed microplates. After 48 h, the cultures were pulsed for 6 h with $1 \mu\text{Ci/well}$ ^3H -TdR (New England Nuclear, Boston, MA) and harvested onto glass fiber filter strips. Incorporation of ^3H -TdR was determined by liquid scintillation spectrometry.

Statistical analysis. In all assays, differences were evaluated by Student's *t* test.

RESULTS

Dose response of BFU-E colony growth to PHA-LCM. Effects of PHA-LCM of adult and cord blood on BFU-E colony formation are shown in Figure 1. The numbers of BFU-E colonies increased at the PHA-LCM concentrations from 0 to 5% and the maximum response was obtained at 5% in every sample. Therefore, this concentration was used for the following BPA assay.

Because PHA-M might be contaminated in the PHA-LCM and the cultures with 5% PHA-LCM might contain at most 0.05% PHA-M, 0.05-0.1% PHA-M was added to the control suspension cultures in order to examine whether these low concentrations of PHA-M had any effects on the increase of the early erythroid progenitor cells. These concentrations of PHA-M did not show any significant effects on the increase of BFU-E colonies (data not shown). Therefore, negative controls were incubated without the addition of PHA-M in the following experiments.

BPA assay. BPA in the PHA-LCM of cord blood and adult blood is shown in Figure 2. The mean value of BPA in the PHA-LCM of cord blood was $37 \pm 13\%$ (mean $\pm$ SD), whereas that of BPA in the PHA-LCM of adult blood was $105 \pm 19\%$. The difference was significant ($p < 0.01$). It is well known that many conditioned media might contain two opposite activities, enhancing and inhibiting. Because BPA in the PHA-LCM might reflect the net activity of positive BPA and possible inhibitors, it was possible that the low BPA in the PHA-LCM of cord blood was due to the presence of inhibitors in the PHA-LCM although the PHA-LCM of cord blood contained as much BPA as that in the PHA-LCM of adult blood. To rule out this possibility, we examined the inhibitory activity of the PHA-LCM of cord blood.

In the first experiments, 5% PHA-LCM of cord blood was added together with adult PHA-LCM in the 2-day incubation to examine whether the amount of inhibitors in cord blood PHA-LCM was sufficient to influence the action of adult PHA-LCM. The cord blood PHA-LCM that showed low BPA by themselves, did not cause significant inhibition in BFU-E colony growth induced by adult PHA-LCM (Table 1).

In the second experiments, the inhibitory effects of cord blood PHA-LCM were examined in one-stage BFU-E colony assay. PHA-LCM of cord blood was used at a concentration of 10% because our preliminary experiments revealed that inhibitory effects of the conditioned medium was generally detected at concentrations higher than 5% in one-stage assay (data not shown). As shown in Figure 3, 10% PHA-LCM of cord blood did not inhibit BFU-E colony growth in the one stage BFU-E

Fig. 1. Dose response of BFU-E colony growth to PHA-LCM of cord blood and adult blood. MNC were cultured with or without PHA-LCM for 2 days and then analyzed for BFU-E colony growth. $\Delta\text{BFU-E}$; (numbers of BFU-E in the cultures) – (numbers of the BFU-E in the control culture maintained without PHA-LCM). C1, C2, C3, cord blood; A1, A2, A3, adult blood.

assay, although neither 10% PHA-LCM of adult blood nor 10% PHA-LCM of cord blood showed an additive BPA activity. It has been reported that exogenously added BPA causes only a slight increase in the numbers of BFU-E colonies in this assay system because crude erythropoietin and endogenously produced BPA from MNC showed submaximal BPA activities (11).

These results showed that the PHA-LCM prepared in this study did not contain an excess of inhibitory factors against BFU-E colony growth and suggested that the low BPA in the PHA-LCM of cord blood was not due to contaminating inhibitors.

CSA assay. The PHA-LCM of cord and adult blood was examined for the ability to stimulate CFU-GM colony growth (Table 2). Maximum CSA response was obtained with 5–10% PHA-LCM and some samples showed inhibitory effect to CFU-GM colony growth at concentrations of more than 10%. Therefore, we used 5% PHA-LCM for detecting CSA. CSA in PHA-LCM is shown in Table 1. There was no significant difference in CSA activities between the PHA-LCM of cord blood and the PHA-LCM of adult blood.

Fig. 2. BPA in PHA-LCM of cord blood and adult blood. BPA is expressed as a percentage of activity to standard PHA-LCM. Horizontal lines denote mean values.

Table 1. Lack of significant inhibitors in cord blood PHA-LCM detected by the two-stage BPA assay system*

Additions to suspension culture	BFU-E derived colonies (per 3×10^5 cells)	
	Experiment 1	Experiment 2
None (control)	38	26
5% adult PHA-LCM	122	93
10% adult PHA-LCM	108	84
5% adult PHA-LCM + 5% other adult PHA-LCM		
No. 1	100	82
No. 2	117	85
No. 3	102	78
5% adult PHA-LCM + 5% cord blood PHA-LCM		
No. 1	106	81
No. 2	101	81
No. 3	96	75
5% cord blood PHA-LCM		
No. 1	ND	54
No. 2	ND	43
No. 3	ND	39

* PHA-LCM of cord blood or adult blood was added to the first stage culture where peripheral blood MNC were cultured with or without PHA-LCM of adult blood for 2 days. BFU-E of the cultures were then evaluated. Each value shows the mean of three replicates. ND; not done.

³H-TdR uptake assay. Mean ³H-TdR uptakes of PHA-stimulated MNC of cord blood and adult blood were $168,000 \pm 46,000$ cpm ($n = 8$) and $157,000 \pm 35,000$ cpm ($n = 7$), respectively. There was no significant difference between these two groups.

DISCUSSION

In this study, we showed that BPA in PHA-LCM of cord blood MNC was significantly lower than that of adult blood MNC. The low BPA in PHA-LCM of cord blood seemed due to the decreased ability of cord blood MNC to produce BPA but not due to contaminating inhibitors. We used Dukes' two-stage assay (11) with a little modification for detecting BPA. This assay system seems better than previously reported one-stage assay (13-15) where crude erythropoietin with or without samples to be examined was added to the culture and the effect of the

Fig. 3. Detection of inhibitors in PHA-LCM to erythroid colony growth. PHA-LCM was added at the final concentration of 10% to mononuclear cells in one-step BFU-E colony assay where 2×10^5 mononuclear cells were incubated in IMDM containing 30% FCS, 1% bovine serum albumin, 5×10^{-5} M 2-mercaptoethanol, 0.9% methylcellulose, and 1 U/ml erythropoietin for 10-12 days. Control, cultured without PHA-LCM; C4-C7, cord blood; A4, A5, adult blood.

Table 2. Colony-stimulating activity in PHA-LCM*

	CFU-GM per 1×10^5 MNC		p
	Cord blood	Adult blood	
Experiment 1	68 ± 32 ($n = 6$)	67 ± 34 ($n = 5$)	NS
Experiment 2	48 ± 32 ($n = 11$)	58 ± 37 ($n = 7$)	NS
Experiment 3	71 ± 29 ($n = 15$)	60 ± 32 ($n = 10$)	NS

* Data were expressed as mean $\pm$ SD. NS; not significant. Only a few colonies (0-3 per 1×10^5 MNC) were induced without PHA-LCM.

samples was compared with that of crude erythropoietin. However, because crude erythropoietin itself has significant BPA, none of these assays seems to be suitable to quantitate BPA. Our modified Dukes' two-stage assay system that did not use erythropoietin in the first stage culture, is thus better than these one-stage assays for quantitation of BPA.

The mechanism of low BPA production in PHA-stimulated MNC of cord blood remains to be elucidated. A possible explanation is a low responsiveness of cord blood MNC to PHA stimulation. However, this explanation seems unlikely because there was no significant difference in ³H-TdR uptake or CSA production between PHA-stimulated MNC of cord blood and that of adult blood. Several studies found a suppressive effect of T cells and diminished function of monocytes of cord blood in other experimental systems (16, 17). These characteristics of cord blood MNC may have some role in low BPA production of cord blood. Further studies are now in progress to investigate the contribution of T cells and monocytes in the low BPA production by cord blood MNC.

It will be of interest to know whether low BPA production of cord blood MNC is responsible to the suppressed erythropoiesis in the neonatal period. Although BPA has been considered to regulate the early stage of erythropoiesis *in vitro*, the physiological role of BPA *in vivo* is still obscure. Further studies are necessary to clarify the relationship of the serum BPA level and BPA production of MNC to erythropoiesis in early infancy.

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