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Perinatal Influences on *in Vitro* B Lymphocyte Differentiation in Human Neonates

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ABSTRACT. *In vitro* differentiation of B lymphocytes present in cord blood mononuclear cell preparations into immunoglobulin secreting cells was studied in 126 neonates with gestational ages (GA) ranging from 20 to 44 wk. Eight infants had a GA less than 27.9 wk, 24 had GA 28–32.9 wk, 30 had GA 33–37.9 wk, 51 had GA 38–41.9 wk, and 13 had GA above 42 wk. B cell differentiation in response to pokeweed mitogen plus hydrocortisone was assessed using a plaque forming cell assay. All neonates had a measurable plaque-forming cell response in this assay. An increased plaque-forming cell response was observed in some neonates in all gestational age groups. The magnitude of *in vitro* neonatal B cell differentiation underwent a continuous and significant ($p < 0.002$) reduction as gestational age increased. The influence of intrauterine growth retardation on *in vitro* B lymphocyte differentiation was studied and compared to gestational age-matched controls with a normal intrauterine growth. Intrauterine growth retardation was not associated with changes in B cell responsiveness. An analysis of perinatal factors revealed that cesarean section, and low 1-min Apgar scores were factors that predisposed cord blood cells to be triggered *in vitro* to produce increased numbers of plaque-forming cells. (*Pediatr Res* 19: 655–658 1985)

Abbreviations

ISC, immunoglobulin secreting cells
AGA, appropriate for gestational age
SGA, small for gestational age
LGA, large for gestational age
MNC, mononuclear cell
PFC, plaque-forming cell
PWM, pokeweed mitogen
HC, hydrocortisone
NK, natural killer
PROM, prolonged rupture of amniotic membranes

Human B-lymphocytes present in cord blood mononuclear cell preparations have been shown to develop into ISC under appropriate culture conditions (1–3). *In vitro* B-lymphocyte differentiation has been assessed only in term newborns who were appropriately grown for their gestational age. We have measured cord blood B-lymphocyte differentiation into ISC's in both premature and full term neonates and in infants who have sustained normal and retarded intrauterine growth. Our study patients range from 20 to 44 wk gestational age and include 62 premature (less than 38 wk gestation) and 64 term (equal to or greater than 38 wk gestation) neonates. Of these neonates 97 were AGA, 17 SGA, and 12 LGA for their gestational age. Our results suggest that B lymphocytes present in cord blood MNC preparations isolated from neonates 20 to 44 wk gestation can be induced *in vitro* to differentiate into ISC's. ISC development was observed in some infants from each gestational age group, with the greatest ISC response being observed in infants from the lowest gestational age group. In analyzing the perinatal influences which may have predisposed cord blood cells to increased ISC development *in vitro*, we found that intrauterine growth retardation was not a significant factor. Although no single parameter consistently led to increased ISC development, cesarean section and low 1-min Apgar scores were associated with a significant increase in ISC production.

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MATERIALS AND METHODS

Heparinized cord blood (30 U heparin per milliliter) was collected from the fetal side of the discarded placenta immediately after delivery as approved by the University Hospital Human Experimentation Committee. The length of gestation was determined by maternal history of the last menstrual period. Where birth weight indicated non-AGA status for a given neonate, a Dubowitz examination was performed by one of the investigators (WBP) to confirm length of gestation (4). All infants were clinically normal with no evidence of an immunodeficiency syndrome. Adult venous samples were collected from healthy volunteers. All blood samples were obtained in accordance with the guidelines established by the Institutional Review Board of University Hospitals of Cleveland.

Mononuclear cell cultures. Peripheral and cord blood MNC's were obtained from heparinized blood samples by standard Ficoll-Hypaque discontinuous gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ). The mononuclear cells were counted with a hemocytometer and identification was confirmed in a random selection of specimens with Wright's staining. Cell cultures for the generation of PFC responses were carried out as described by Fauci *et al.* (5). Five $\times 10^5$ mononuclear cells in 1 ml of RPMI 1640 medium containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10% heat inactivated fetal calf serum (FCS), and 20 μl/ml of 8.4% sodium bicarbonate were incubated in 12 × 75 mm round bottom plastic tubes (Falcon no. 2054).

Cell cultures were stimulated with PWM (GIBCO, 2.5 μl/ml), with HC (HC sodium succinate): Solu-Cortef Upjohn; 10^{-5} M), or with PWM and HC together. Optimal mitogen concentrations were established in preliminary studies (3). Cultures were maintained at 37° C in 5% CO_2 in air at 100% humidity for 5 days prior to indirect PFC assay.

Indirect protein A PFC assay. PFC's were enumerated by the method described by Fauci *et al.* (5). Sheep red blood cells were

Table 1. *PFC response of mononuclear cells from neonates of different gestational age (mean ± SE)*

Group	n	Gestational age (wk)	Body wt (kg)	PFC/10^6 MNC PWM (alone)	PFC/10^6 MNC PWM + HC
I	8	24.5 ± 0.9	0.7 ± 0.1	1625 ± 716	9436 ± 5983
II	24	30.0 ± 0.3	1.3 ± 0.1	1408 ± 534	13579 ± 3859
III	30	35.4 ± 0.3	2.4 ± 0.1	662 ± 305	8893 ± 2131
IV	51	39.3 ± 0.1	3.0 ± 0.1	643 ± 197	4186 ± 800
V	13	42.3 ± 0.1	3.6 ± 0.2	365 ± 239	4915 ± 1123
I-V	126	36.0 ± 0.4	2.4 ± 0.1	827 ± 192	7505 ± 1058*

* Value of groups I, II, and III is significantly greater than groups IV and V (term neonates): $p < 0.002$.

Table 2. *PFC response related to intrauterine growth status (mean ± SE)*

	n	Gestational age (wk)	Body wt (kg)	PFC/10^6 MNC PWM (alone)	PFC/10^6 MNC PWM + HC
AGA*	97	35 ± 0.5	2290 ± 96	791 ± 182	8364 ± 1334
SGA*	17	38.6 ± 0.6	2008 ± 96	625 ± 522	4685 ± 1380
LGA*	12	40 ± 0.5	4124 ± 74	1404 ± 1217	4559 ± 1293

* AGA, SGA, and LGA indicate neonates 10th to 90th (appropriate), less than 10th (small) and greater than 90th (large) percentile birth weight for gestational age, respectively.

coupled to purified protein A (SpA; Pharmacia Fine Chemicals) by using a chromium chloride technique (6). Cell cultures were harvested, washed, and resuspended in cold RPMI 1640 medium. Plastic Petri dishes (60 × 15 mm; Falcon no. 1007) were precoated with 0.7% agarose (Accurate Chemicals, Hicksville, NY) in RPMI 1640. An ultrathin layer consisting of 100 μl of resuspended cultured cells, 0.85 ml of agarose in RPMI 1640, and 50 μl of a 15% suspension of SpA-coated SRBC was poured evenly over the agarose-precoated Petri dishes, cooled, and incubated at 37° C for 2 h. The plaques were developed by layering 1 ml of a 1:100 dilution of the IgG fraction of a rabbit anti-human polyvalent Ig (Cappel Labs, Cochranville, PA) onto the Petri dishes. The plates were then incubated overnight at 37° C. The antiserum was removed, and 1 ml of guinea pig complement (M.A. Bioproducts, Wakersville, MD; 1:40 in barbital buffered saline) was added. After a 1-h incubation the complement was removed and plaques were read under indirect light at 4 × magnification. Data are expressed as PFC/10^6 cultured cells.

Statistical analysis. Results are presented as the mean and SEM. A standard t test was employed.

Data recorded concerning each infant included gestational age, birth weight, sex, Apgar score at 1 min, and classification as to LGA, SGA or AGA *in utero* (7). Recorded maternal data included age and gravidity, mode of delivery, presence or absence of ruptured amniotic membranes greater than 24 h prior to delivery, and presence or absence of meconium stained amniotic fluid. These clinical parameters were then compared with the lymphocyte differentiation data from the total population of neonates. The number of PFC developed per 10^6 cord blood mononuclear cells in response to PWM alone and PWM plus 10^{-5} M hydrocortisone was measured in 97 appropriately grown *in utero* (AGA), 17 growth retarded (SGA), and 12 LGA neonates (5). For data analysis, the 126 neonates were divided into five gestational age categories including eight infants 20 to 27 wk, 24 infants 28 to 32.9 wk, 30 infants 33 to 37.9 wk, 51 infants 38 to 41.9 wk, and 13 infants 42 to 44 wk.

RESULTS

PFC's were detected in MNC cultures from all infants in response to PWM plus HC, whereas 50% of cord blood cultures had a minimal response to PWM alone of less than 100 PFC/10^6 MNC (Table 1). The PFC response of the cord blood lymphocytes to PWM plus hydrocortisone from all 126 neonates was significantly correlated with the PFC response to PWM alone ($p < 0.001$). Adult MNC cultures ($n = 15$) stimulated with PWM produced a significant level of PFC's (17,083 ± 4356, mean ± SE PFC/10^6 MNC) that was increased to a maximum response (59,949 PFC/10^6 MNC) in the presence of both PWM and HC (data not shown). No adult or neonatal culture produced PFC's with HC alone.

The mean number of PFC's generated in response to PWM plus hydrocortisone significantly decreased ($p < 0.002$) as the gestational age increased from 20 to 44 wk (Table 1). Further, the mean PFC response measured at birth was significantly greater ($p < 0.002$) in preterm infants (≤37.9 wk gestation) than it was in more mature neonates (≥38 wk gestation).

Although PFC responses were observed in all gestational age groups, not all individuals in each group had a large response. If a range of response is established for the term neonates (group IV) of 0 to 10,000 PFC/10^6 MNC (mean ± 1 SD of group IV), it was observed that 11.4% of group IV had PFC responses above 10,000 PFC/10^6 MNC, whereas 40.0, 38.9, and 26.1% of groups I, II, and III had PFC responses greater than 10,000 PFC/10^6 MNC.

Intrauterine growth retardation as a reflection of intrauterine placental dysfunction was not significantly related to the increased PFC value observed in the younger gestational age groups as shown in Table 2. Therefore, other parameters that might influence this PFC response were evaluated. Neither maternal age and/or gravidity, nor the infant's sex, PROM, or the presence or absence of meconium in the amniotic fluid were significantly correlated with the PFC response measured. On the other hand, the mode of delivery and Apgar score at 1-min after delivery were related to significantly increased PFC responses (Table 3).

DISCUSSION

Cord blood B-lymphocytes unlike adult B cells present in isolated MNC preparations are normally unable to differentiate into ISC when cultured in the presence of PWM alone. The data on ISC development in neonatal B lymphocytes from cord blood MNC preparations presented here were obtained using PWM plus HC stimulation of MNC and detection of ISC in a reverse plaque assay using polyvalent anti-Ig as the developing antibody. When MNC from cord blood are cultured in the presence of PWM alone, minimum levels of PFC are produced, unlike the response seen with adult MNC. Activators such as live Epstein-Barr virus (1, 8), killed *Staphylococcus aureus* (3, 9, 10) or a combination of *S. aureus* and PWM (2) have been used to induce

Table 3. *Influence of cesarean section and low 1-minute Apgar score on neonatal PFC response (mean ± SE)*

Group	*n*	Gestational age (wk)	Body wt (kg)	PFC/10^6 MNC	
				PWM (alone)	PWM + HC
I. Vaginal delivery	87	35.8 ± 0.6	2415 ± 111	669 ± 188	6064 ± 1028
					$p < .05$
Cesarean section	39	35.8 ± 0.7	2337 ± 163	1180 ± 459	10,719 ± 2482
II. Apgar ≥ 7	82	37.7 ± 0.4	2671 ± 94	685 ± 250	5442 ± 826
					$p < .008$
Apgar ≤ 6	44	32.3 ± 0.9	1870 ± 171	1092 ± 294	11,348 ± 2528

adult levels of PFC production and intracytoplasmic immunoglobulin production from cord blood MNC cultures. We have found that stimulating cord blood MNC with PWM plus HC will amplify the response due to PWM alone. Although the response is not equivalent to adult levels, the cord blood PFC response is amplified sufficiently to detect significantly elevated responses. HC has been noted to enhance immunoglobulin synthesis by PWM-stimulated adult human lymphocytes (11). A direct effect of corticosteroids on the B-lymphocyte has been suggested (12), although an effect on regulatory T-cells has also been described (13). HC will inhibit both Interleukin 1 (14) and Interleukin 2 (15, 16) production to certain degrees. Both Interleukin-1 and Interleukin-2 are potent regulator proteins in the induction of the immune response because of their ability to control proliferation and the production of differentiation factors. A decrease of Interleukin-2 without a decrease of B-cell growth-promoting activity will enhance B cell proliferation (17). A combination of the effects of HC may partly explain the enhancement of PFC production in adults. Whether a similar mechanism may account for the enhanced PFC development in the neonate needs to be elucidated. In this investigation, we used the enhancement of neonatal PFC's to study the influence of perinatal events on the development of immunoglobulin secreting cells *in vitro*.

Our data show that mononuclear cells isolated from the cord blood of neonates of varying gestational ages can be induced under appropriate *in vitro* conditions to develop a significant number of ISC's. Although each gestational age group was able to show this response, the greatest level of ISC was observed in the early gestational age groups. This increase in *in vitro* ISC development in the most immature groups of neonates was puzzling, since as the fetus matures the capacity for immunoglobulin synthesis increases. Since it was also observed that some individuals from each group produced significant levels of PFC's but others from the same group did not, parameters other than neonatal maturity were sought as an explanation for increased PFC development in the LGA infant.

When the PFC responses of the entire population were evaluated it was noted that some perinatal events were associated with an increased ability of circulating B lymphocytes to develop into ISC *in vitro*. Delivery by cesarean section and Apgar scores of 6 or less at 1-min were each associated with a significantly greater PFC response than that measured in neonates not exposed to these perinatal influences. Each perinatal factor regarded individually could be shown to have an influence on increased PFC development. However, these perinatal factors frequently occur in the same individual, and it becomes difficult to attribute increased PFC development to any single factor. Premature infants are more likely to have low Apgar scores at birth than term infants. Therefore, increased PFC responses are not solely the result of LGA. Term infants associated with these perinatal factors also had higher responses than term infants not exposed to them.

Whether a constant mechanism predisposing neonatal B lymphocytes to increased *in vitro* development into ISC is present in neonates delivered by cesarean section and in infants with low 1-min Apgar scores cannot be defined from the data presented herein. Further studies will be necessary to elucidate this question.

The significantly increased PFC response among the neonates delivered by cesarean section as compared to vaginally delivered infants is of interest. Neonatal lymphocyte mitogen induced proliferation has recently been noted to be significantly increased in cesarean section versus vaginally delivered infants (18). In contrast, NK cell activity has been reported to be decreased in surgically delivered neonates (19). These observations support a mechanism of immunoregulation by NK cells (20). Abruzzo and Rowley (21) have recently reported that NK cells promote early termination of an ongoing primary immunoglobulin M PFC response and that the target for NK cells is a population of accessary cells that has interacted with antigen and is necessary for sustaining the antibody response. We are now exploring the effect of cesarean section with and without labor and of vaginal delivery on several parallel immune functions in order to gain further insight into this observation.

Suppressor T-cells and related suppressor factor mechanisms have been invoked to explain the diminished immune responsiveness of the neonate (22–24). Several studies using monoclonal T-cell markers have shown that the neonate has normal numbers of circulating T-cell subpopulations (25–27). These reports have not evaluated the influence of perinatal events on T-cell subpopulations. Preliminary studies in our laboratory have revealed a significantly higher proportion of OKT8 positive T-lymphocytes in newborns delivered after maternal labor, as compared to newborns delivered by elective cesarean section in absence of labor (Pittard III WB, unpublished observation). Although one report suggests that the suppressor activity in the newborn is a function of lymphocytes expressing the OKT4+ (helper/inducer) phenotype, the suppression exerted by this subpopulation is not greater than the suppression caused by the OKT8+ (suppressor) population in adults (28). Whether the observed changes in T-lymphocyte subpopulations correlate with the observed increased lymphocyte proliferative response and increased PFC response in neonates delivered by cesarean section needs to be determined.

The increased PFC response seen in infants with low Apgar scores may be due to factors different from those operative in cesarean section. Stress is likely to be present in some of these neonates. It is interesting to postulate that stress may have an effect on immunoregulatory cells, producing changes in the immune response capabilities which may be detected through functional testing as done in this investigation.

Intrauterine and neonatal infections can lead to an increased immunoglobulin production *in vivo* (29). Our study population did not include neonates with clinically recognizable intrauterine infection. PROM is associated with an increased incidence of neonatal infection and sepsis (30). As a single perinatal factor, PROM was not associated with increased PFC development in our study. This could be due to the relatively short lapse of time between a possible antigen exposure caused by PROM and the collection of the cord blood sample used in our assay. On the other hand, our PFC assay does not detect *in vivo* B-cell differentiation and immunoglobulin production. Rather, it measures the extent neonatal B-lymphocytes respond to *in vitro* stimulation by polyclonal-B-lymphocyte activators. The conditions that

lead to an increased B-lymphocyte responsiveness *in vitro* do not appear to be related to *in vivo* exposure of T- and B-lymphocytes to bacterial antigens.

In summary our results indicate that active *in vitro* development of PFC's can be observed in some neonates at all gestational ages beyond 20 wk. Several clinical settings are identified which are associated with increased neonatal PFC development *in vitro*, but none of these perinatal factors consistently leads to increased PFC responsiveness. The higher PFC levels at lower gestational age may reflect a higher incidence of such predisposing factors in premature newborns.

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