Frequency and Cytokine Phenotype of Blood T Cells from Premature Infants Responding to Staphylococcal Enterotoxin B

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ABSTRACT

The responder cell frequency (RCF) of premature (<1900 g birth weight) infants' blood lymphocytes, which proliferate in cultures stimulated by staphylococcal enterotoxin B, falls from 1:3400 to about 1:8000 during the first 2 wk of life. Term infants, in contrast, show no fall in RCF. The reduced RCF in the premature infants affected cells that make interferon- γ more than cells making IL-4. The reduced RCF was accompanied by a fall in the fraction of V β 3⁺ T cells that entered cell cycle in stimulated cultures. The RCF of premature infants' T cells was increased in cultures supplemented with irradiated monocytes from adults. Addition of IL-4 (but not IL-2, IL-6, or indometha-

cin) increased the RCF and fraction of cells entering cell cycle of the premature infants. The data suggest that postnatal environmental factors limit the ability of premature infants' monocytes to support a T-cell response to staphylococcal enterotoxin B *in vitro* and that this limitation is overcome by adding IL-4. (*Pediatr Res* 37: 455–459, 1995)

Abbreviations

RCF, responder cell frequency
SEB, staphylococcal enterotoxin B
IFN, interferon

Premature infants make lower antibody responses to influenza (1, 2) and tetanus (3, 4) immunization during their first 2 y of life compared with age-matched children born at full term. This finding may be clinically relevant because premature infants have an increased mortality and morbidity from virus infections of the respiratory tract compared with infants born at full term (5, 6). Our previous finding that premature infants had lower numbers of influenza antigen-specific T cells than term infants after immunization (2) raises the possibility that impaired T-cell function might limit the antibody response to influenza. The frequency of T cells in blood that respond to influenza antigen is, however, too low for studies of influenza-specific T cells to shed light on the development of T-cell responses in very premature infants. To develop an alternative, antigen-nonspecific, model to quantitate the *in vitro* responses of blood lymphocytes from human newborns and adults, we determined the frequency and cytokine profiles of blood T cells responding *in vitro* to SEB (7). SEB is particularly suited to this purpose as the structure is known (8), together with mechanism by which it binds to MHC molecules (9). Furthermore, the stimulation of T cells is highly dependent on acces-

sory cells or their cytokines (10, 11), and the major subset of responding cells can be identified by cell surface expression of the V β 3 family of T-cell receptors (12). Both IFN- γ -secreting (TH1-like) and IL-4-secreting (TH2-like) T cells respond to SEB (13).

Our previous studies of premature infants had shown a decreased proliferative response to SEB by blood lymphocytes at 1–2 y of age compared with controls born at term (2). The studies reported here were undertaken to determine whether responses to SEB would be reduced in the neonatal period and, if this were the case, whether there would be differences in the fraction of cells activated and their cytokine profile.

METHODS

Human subjects. Premature infants born at <32 wk of gestation weighing <1900 g were identified through the Pediatric Clinical Research Center. Blood lymphocytes were recovered from blood samples obtained between 10 and 20 d of age (mean 14 d) for routine biochemical monitoring. These samples had been anticoagulated with preservative-free heparin, and the cells were always processed on the same day as they were drawn. The subjects were studied anonymously, and institutional review board exemption from consent form use was obtained. Blood was also obtained from the placenta of healthy infants born at term. Term infants were defined as born at >38 wk of gestation weighing >2.5 kg. They, and the

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healthy adults whose blood was used as a source of monocytes, were enrolled subject to informed consent.

Lymphocyte cultures. Limiting dilution cultures were performed as described in Hayward *et al.* (14) except that the replicates of 24 wells contained between 10 000 and 300 cells per well and were incubated for 8 d. SEB was obtained from Sigma Chemical Co. (St. Louis, MO) and added at 1 $\mu\text{g/L}$, and cultures were supplemented with 0.001–100 U/mL recombinant cytokines where shown. Cytokine sources were IL-2: catalog no. 07050 from Amgen, Thousand Oaks, CA, and IL-4: catalog no. 4212-55 and IL-6: catalog no. 4215-00, both from Interger, Purchase, NY. Indomethacin (Sigma Chemical Co.) was dissolved in DMSO and added to cultures at a final concentration of 0.5 μM . The wells were pulsed for 6 h with 0.25 μCi of [^3H]thymidine, harvested onto glass fiber, and counted on a Betaplate (LKB Wallac, Turku, Finland) reader with direct data transfer and reduction by χ^2 minimalization (15).

To supplement wells with adult adherent cells, adult blood mononuclear cells were obtained as previously described (14). The cells were suspended in RPMI 1640 medium with 1% human serum at 10^6 cells/mL, and 10^5 cells were added to the wells of 96-well plates. Nonadherent cells were washed out of the wells after 1 h, and the plate was irradiated with 3000 rad from a ^{60}Co source. Visual examination indicated that these wells contained about 400 adhered cells.

Cultures for cell cycle studies were of whole blood diluted 1:20 with RPMI. Cells bearing T-cell receptor β -chains of the V β 3 family were identified by staining with FITC-conjugated antibody (T Cell Diagnostics, Cambridge, MA). These cells were fixed in 80% methanol, RNase-treated to avoid green staining of RNA, and then stained with propidium iodide. The cell cycle status of the V β 3 cells was determined by two-color immunofluorescence on an EPICS C (Coulter Electronics, Hialeah, FL) with a logarithmic green scale and linear red scale as previously described (16).

ELISA for cytokines. IL-4 and IFN- γ were measured by ELISA as previously described (7). Briefly, Immulon II plates (Dynatech, Chantilly, VA) were coated at 4°C overnight with Genzyme (Cambridge, MA) antibodies to human IFN- γ (catalog no. 1598-00, diluted 1:300) or IL-4 (catalog no. 1842-01, diluted 1:400) in sodium bicarbonate buffer (pH 8.4). The plates were blocked with PBS-gelatin (1 mg/mL) at 37°C for 2 h. Then 20 μL of the limiting dilution culture supernatants were added to the wells. The assay was calibrated with recom-

binant cytokines, 0.06, 0.6, and 6 U/well for IFN- γ and 0.8, 4, and 20 U/well for IL-4. After 2 h, the wells were washed with 0.075 M PBS-Tween four times. The secondary antibodies (rabbit anti-human polyclonal anti-cytokine antibodies) to IFN- γ and IL-4 were added at 1:500 diluted in PBS with 1% FCS and held at 4°C overnight. After four washes, a biotin-conjugated affinity-purified goat anti-rabbit IgG (Organon Teknika Corp., West Chester, PA) diluted 1:5000 in PBS and 1% FCS was added, and the plates were incubated at 37°C for 2 h. After four washes, alkaline phosphatase-conjugated avidin, diluted 1:5000 in TBS, was added and incubated at 37°C for 45 min. The substrate was *p*-nitrophenyl phosphate and glycine buffer (Sigma Chemical Co.) which was prepared and added as directed. The plate was incubated at 37°C, with the OD read on a Dynatech MR 600 Microplate Reader.

Statistics. RCF results were log-transformed for analysis. Comparisons between groups were by two-tailed Wilcoxon two-sample tests.

RESULTS

Limiting dilution analyses of SEB RCF. Blood lymphocytes from newborns and 2-wk-old infants, premature and full term, were cultured in U-bottom wells for 8 d with and without SEB. The RCF calculated by χ^2 minimalization (Table 1) is significantly lower for newborns and infants compared with adults, in keeping with our previous report. There were, however, no differences in the RCF to SEB by cord blood lymphocytes obtained from premature and term newborns when tested by Spearman rank correlation test. Two weeks after birth, the RCF of lymphocytes from the premature infants is significantly lower than the full-term controls ($p < 0.03$, two-tailed test). Inasmuch as there had been no difference in RCF between premature and term newborns, prematurity alone is unlikely to account for the difference in RCF at 2 wk. The response to SEB is highly dependent on monocytes to bind and present the ligand, so we compared the number of blood monocytes in the premature and term infants identified by a differential white cell count, but no difference was found (Table 1).

To determine whether impaired accessory cell function contributed to the reduced RCF to SEB of the premature infants, limiting dilution cultures were supplemented with irradiated adherent cells obtained from unrelated healthy adults. The frequency of cells responding to SEB in these cultures was determined as the increment over cultures with allogeneic

Table 1. Frequency of blood lymphocytes responding to SEB falls in the 2 wk after premature birth

Subjects	Age	n	Mean RCF (1:x)	1 SD range (1:x)	Monocytes/ μL
Term	Newborns	12	3135	2153–4191	1200 $\pm$ 560
Premature	Newborns	10	3350	1900–5150	ND*
Adults	>20 y	8	393	120–1737	ND
Term	2 wk	8	4520	2290–6700	960 $\pm$ 470
Premature	2 wk	8	9400	7380–12500	1100 $\pm$ 490

RCF results are the frequency of cells responding by proliferation, estimated from limiting dilution cultures. Differences between adults and newborns, either term or premature, are statistically significant ($p < 0.05$), as are differences between the term and premature infants. The monocyte count was calculated from the white cell count and the percent monocytes seen in a manual count, ± 2 SD.

* Not determined.

Table 2. The RCF to SEB of premature infants' lymphocytes is increased in the presence of irradiated adult adherent cells

Subjects	Number tested	Added adhered cells	Mean RCF (1:x)	1 SD Range (1:x)
Prematures	3	None	8800	6220–9460
Prematures	3	Adult	3950	2250–4680
Newborns	4	None	3400	2160–4480
Newborns	4	Adult	3130	2400–4100

Premature infant cells were from subjects with a mean age of 14 d. Newborns were term infants. The frequency of cells responding to SEB was calculated as the increment in RCF over wells containing allogeneic monocytes alone.

monocytes but without SEB. The results (Table 2) indicate that the RCF of the premature infants was increased to levels found in term controls in these cultures. The response to SEB by term infants' cells was not increased by the presence of adult monocytes.

The ability of adult monocytes to increase the RCF to SEB argued against *in vitro* suppression of the T-cell response by soluble mediators such as prostaglandin E₂. To exclude a role for prostaglandin E₂ suppression, cultures were supplemented with 0.5 μ M indomethacin, a cyclooxygenase inhibitor. No increase in the response of premature infants' T cells to SEB was found in these cultures (data not shown).

Cytokine phenotype of cells responding to SEB. To determine whether the reduced response of the premature infants when tested at 2 wk of age affected mainly TH1- or TH2-like cells, we measured IFN- γ and IL-4 in the supernatants of the limiting dilution culture wells. Responder wells were defined as those with cytokine levels above the (mean $\pm$ 2 SD) for the unstimulated controls. Applying the χ^2 minimalization procedure to these data gives an estimate of the precursor frequency of IFN- γ - and IL-4-making cells. When applied to cord blood cells stimulated for 5 d with SEB, this method shows a mean of 1:98 000 cells as positive for IFN- γ and <1:10⁵ as positive for IL-4. The results for the 14-d-old infants (Table 3) show a statistically significant reduction in IFN- γ -making cells among those born prematurely. Fewer cells made IL-4, and although there was a trend toward lower frequencies of responders among the premature infants, it did not reach statistical significance.

Table 3. Frequency of IFN- γ and IL-4 making cells in SEB-stimulated cultures of infants 14 days after premature or term birth

Subjects (n)	End point	Mean RCF (1:x)	1 SD range (1:x)
Term infants (8)	Proliferation	4,300	2 260–8 600
	IFN- γ	53,360*	38 110–74 700
	IL-4	60,840	43 460–85 180
Premature infants (6)	Proliferation	8,890	4 420–17 000
	IFN- γ	84,850*	60 610–118 790
	IL-4	71,870	51 340–100 620

Wells were defined as positive for IFN- γ or IL-4 when their level was $>$ mean + 2 SD for the parallel unstimulated controls.

* The frequency of IFN- γ making cells in premature and term infants differs significantly ($p < 0.05$).

Cell cycle entry in SEB stimulated cultures. The reduced RCF of the premature infants might have resulted from reduced activation of T cells in the cultures. To confirm this possibility, we measured the fraction of V β 3 cells that entered cell cycle after SEB stimulation. The results (Fig. 1) show a daily increment in the percentage of V β 3 cells that have moved out of G₀ into cell cycle through the first 4 d of culture. The percentage of V β 3 cells remained between 4 and 8% for the first 3 d of culture but from the fourth day of culture onward increased, presumably due to the replication of this subset. The fraction of V β 3 cells in cycle after 3 d of culture is similar in term newborns (32.1%) and premature infants (30.3%) when measured in the first days of life. However, the percentage of the premature infants' V β 3 cells that entered cell cycle in SEB-stimulated cultures fell to a mean of 19% (range 13–25%) 14 d after birth. This was significantly less than the 30.3% of V β 3 cells that were in cycle from infants aged 14 d who had been born at term (Fig. 2). Because the activation of naive T cells by SEB requires a range of second signals, including IL-2, IL-4, and IL-6, we measured cell cycle entry in the presence of added cytokines. The results (Fig. 2) indicate that 50 U/mL of IL-4 was able to increase the percentage of the premature infants' V β 3 cells entering cell cycle to control levels. Neither IL-2 nor IL-6 had an effect at concentrations up to 100 U/mL (not shown).

The effect of IL-4, together with IL-2 and IL-6, was also tested in limiting dilution cultures with SEB. The results show a statistically significant increase in response by premature infants' T cells with IL-4 (Table 4) but no influence of IL-2 or IL-6 at levels up to 100 U/mL. Addition of IFN- γ at 100 U/mL also had no effect (not shown). The RCF of the term infants appears to fall in the presence of IL-2 concentrations >10 U/mL. This is an artifact caused by an increase in thymidine uptake in the unstimulated control wells, from which the mean $\pm$ 3 SD cutoff point for responder wells is calculated.

DISCUSSION

Evidence for impaired cell-mediated immune responses after premature birth is indirect and at best inconclusive when the stressful environment of neonatal intensive care is taken into account. The number of studies of T-cell function of premature infants has been limited by the very small volumes of blood

Figure 1. Percentage of newborns' V β 3⁺ T cells in cell cycle on different days after stimulation by SEB. Ranges shown are $\pm$ 1 SD.

Figure 2. Cell cycle entry by premature infant Vβ3 T cells is increased when 50 U/mol IL-4 is added to cultures. The difference is statistically significant ($p < 0.05$ by Wilcoxon two-sample statistic, $n = 8$ for premature infants and control groups, respectively). Ranges shown are ± 1 SD.

Table 4. Effects of added cytokines on RCF to SEB of lymphocytes from infants 14 d after premature or full-term birth

Stimulus	Preterm infants		Term infants	
	RCF 1:x	Range	RCF 1:x	Range
SEB	8700	7 620–9 850	3400	2430–3870
SEB + IL-2	7900	6 800–10 200	6620	2400–4620
SEB + IL-4	4400*	4 020–5 530	3300	2780–3770
SEB + IL-6	8100	7 000–10 100	3500	2870–4450

Results are from five premature and five term infants. Data shown were obtained with 50 U/mL cytokines.

* The preterm response to SEB in the presence of IL-4 is significantly greater than the control without cytokine or in the presence of IL-2 or IL-6 ($p < 0.05$).

available, and the approaches used have been relatively non-specific. Phenotyping studies indicate that premature infants have as many T cells as full-term controls (17, 18). Proliferation of premature infants' T cells obtained on the day of birth and stimulated by phytohemagglutinin were similar to adults in some studies (19, 20) and low in others (21), but possible contributions of impaired accessory cell function or deficiency of a subset of T cells have not been examined. The wider use of immunization to prevent infectious diseases after premature birth has refocused attention on immunity development (or impairment) in ex-premature infants. Particularly important in this context is the duration of any impairment in T-cell response. Bussel *et al.* (22), for example, found a modest impairment of response to phytohemagglutinin in premature infants which was greatest at birth and persisted for at least the first 2 mo of life.

Our own studies point to a reduced frequency of influenza-specific T cells (accompanied by lower antibody responses) to influenza in a population of premature infants aged 1–2 y who were compared with age-matched healthy controls born at term (2). This result suggested that premature birth could diminish the antibody response to immunization for as long as 24 mo after birth. Analysis of T-cell development in the first weeks of life requires ligands, such as SEB, which stimulate a large number of cells. By combining SEB stimulation with quantitative end points, such as limiting dilution cultures, and cell cycle analysis, we have been able to explore, using very small volumes of blood, the activation of cells defined by their Vβ expression. A weakness of SEB as a model activating agent is

that no processing by the antigen-presenting cells is required, that binding to class II MHC occurs outside the antigen-binding cleft, and binding to the T-cell receptor is through the β -chain rather than the $\alpha\beta$ combination (9). Nevertheless, live antigen-presenting cells are required to support a proliferative response, because the response to SEB is accessory factor dependent (11). Newborns' monocytes have mature antigen-presenting cell function (23), and the reduced response of newborn T cells to SEB is at least in part associated with their CD45RA phenotype (24) and the intrinsically lower response of this subset to SEB (25).

The frequency of term infants' Vβ3⁺ T cells that entered cell cycle or proliferated in SEB-stimulated cultures remained roughly constant during the first weeks of life. Response by the premature infants' Vβ3⁺ cells fell significantly during the same time period, whether measured as the frequency of cells entering cell cycle or the frequency proliferating in limiting dilution. The frequency of term infants' cells making IFN- γ at 2 wk of age is higher than at birth (1:53 000, compared with the 1:98 000 we found for newborns). Because $< 1:10^5$ newborns' mononuclear cells made IL-4 in cultures stimulated by SEB, the frequency of wells positive for this cytokine almost doubled in the first 2 wk of life. The number of cells positive for IFN- γ and IL-4 detected in the limiting dilution cultures reported here is less than that we previously reported as positive in an Elispot assay (7). It is likely that this difference results from proliferation by cells responding to SEB in the Elispot assay, as shown by our measurements of Vβ3⁺ cells in SEB-stimulated cultures in the present study (Fig. 1). In limiting dilution cultures the estimate of frequency of precursors of cytokine-making cells is less open to increase as a result of proliferation.

It is likely that reduced availability of accessory cells or their factors contributed to the fall in SEB response of the premature infants in the first 2 wk of life, as their cells responded as well as term infants' cells in the presence of adult monocytes. The reconstitution that followed the addition of MHC-mismatched adult monocytes is open to multiple interpretations, including the production of cytokines by premature infants' T cells responding to the unrelated histocompatibility antigens of the adults' monocytes. Although some alloantigen stimulation undoubtedly occurred, it did not obscure the response to SEB. The small proportion of allogeneic monocytes (about 400 cells) that was cultured with 10^5 responder MNC may have contributed to our continuing ability to detect a response to SEB. Together with the increase in premature infants' Vβ3⁺ that entered cell cycle in the presence of SEB, the result argues against an active suppression by the newborns' monocytes of T-cell responses to SEB. Nonspecific suppression by the premature infants' monocytes, such as the autocrine suppression of monocyte cytokine production by prostaglandin E₂ (26), is also unlikely, because the reconstitution provided by adult monocytes occurred in the continuing presence of the newborns' monocytes. This interpretation is supported by our failure to increase the response of premature infants' T cells to SEB by adding indomethacin to the cultures.

Of the cytokines that were tested, only IL-4 was able to restore the premature infants' response to the levels of the term

controls, whether the end point was the fraction of V β 3 cells entering cell cycle or the RCF. The mechanism by which IL-4 boosted the response of premature infants' MNC to SEB remains to be established. IL-4 is a multifunctional cytokine that modulates the cytokine profile of T-cell responses, amplifying the IL-4 component and tending to diminish the IFN- γ component (27). IL-4 can act directly on T cells, including naive T cells (28), to maintain their proliferation in culture. IL-4 also acts on monocytes to modulate their expression of integrins (29) and to increase their expression of cell surface HLA-DR and DP molecules (30). Any of these effects could contribute to an increase in the frequency of T cells responding to SEB. The principal conclusion of the reconstitution experiments, whether by adult monocytes or IL-4, is that the premature infants' V β 3 cells retained the potential for response.

In summary, our results provide evidence for an acquired defect in premature infants' blood monocytes that limits their ability to present a T-cell ligand, SEB, to V β 3⁺ T cells. The nature of the defect, and its duration, remain to be established. Prospective studies will be required to determine whether impairment of T-cell responses to SEB in the first weeks of life correlates with any alteration in susceptibility to infection or ability to respond to immunization.

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