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Dysregulation of IL-13 Production by Cord Blood CD4$^+$ T Cells Is Associated with the Subsequent Development of Atopic Disease in Infants

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ABSTRACT

Early intervention strategies in allergic diseases will be dependent on identification of newborns at high risk for later development of atopic disease. In this cohort study of 106 neonates, we investigated whether cytokine production property and responsiveness to IL-12 of neonatal CD4$^+$ T cells were associated with the subsequent development of atopic disease and whether a skewed cytokine production property was intrinsic to helper T cells. To exclude the effects of contaminating cells, highly purified cord blood CD4$^+$ T cells were stimulated with anti-CD3 MAb and recombinant B7-2 molecule in the presence or absence of IL-12. Production of IL-13 and interferon-γ was determined by ELISA. The infants were assessed at 12 mo for the development of atopic diseases. CD4$^+$ T cells of neonates who manifested allergic symptoms (atopic group) produced higher levels of IL-13 compared with those of the nonatopic group in both the presence and absence of IL-12. No significant difference was noted between the two groups with respect to interferon-γ production. Moreover, higher IL-13 production was also observed in neonates with chronic eczema than those with short-term eczema. Our data suggest that increased production of IL-13 by neonatal CD4$^+$ T cells is a useful marker of newborns at high risk for subsequent development of atopic diseases and that an intrinsic abnormality of CD4$^+$ T cell is associated with the pathogeneses of atopic disease, especially atopic dermatitis in infants. **(*Pediatr Res* 51: 195–200, 2002)**

Abbreviations

CB, cord blood
IFN, interferon
APC, antigen-presenting cells

Inasmuch as most atopic diseases first appear in the very young and sensitization is known to occur in early life (1, 2), prediction of the high-risk group as soon after birth as possible is important to design effective prevention programs for atopic diseases. Several studies have focused on Th1/Th2 type cytokine production by CB cells and showed that a low IFN-γ-producing capacity was associated with a positive family history of allergy and/or subsequent development of atopic disorders (3–6). However, it is not clear at present whether the reduced IFN-γ production by CB mononuclear cells is intrinsic to T cells or due to coexisting cells such as natural killer cells and monocytes (7). The differentiation of naive T cells into Th1/Th2 effectors is regulated by several factors including genetic background (8). In mice, the effects of genetic background on Th phenotype development have been shown to reside within T cells, but not APC (9). Humoral environments during delivery, such as prostaglandins, may modulate the function of APC resulting in reduced IFN-γ production (10). Thus, highly purified CB CD4$^+$ T cells should be used to evaluate the intrinsic differentiation property of naive T cells into Th1/Th2 effectors.

IL-13 shares a number of biologic activities with IL-4 and plays an important role in IgE synthesis (11). The exaggerated production of IL-13 is observed in asthma and atopic dermatitis (12, 13). Although IL-13 is produced mainly by activated Th2, native T cells also produce relatively high levels of IL-13, which are affected by endogenous IL-4 (14). In this study, we have measured IL-13 and IFN-γ production by highly purified CB naive CD4$^+$ T cells, and discuss its predictive value for the development of future atopic diseases in infants.

METHODS

Study subjects. CB samples were consecutively collected from 106 term babies (>37 wk gestation) (62 males, 44 females) born between October 1, 1998, and February 14, 1999, without perinatal complication at an obstetric department. All samples contained <3.6 mg/L of IgA indicating no

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considerable contamination of maternal blood (4). The family history of bronchial asthma, allergic rhinitis, and atopic dermatitis in the parents was obtained from their mothers. Infants were clinically assessed for symptoms of atopic disease at 4, 7, and 12 mo by medical practitioners without notification of the results of cytokine production assay. A questionnaire asked for details about the presence of atopic disease—the diagnosis of atopic dermatitis and bronchial asthma by physicians, presence of an eczematous rash and its site and duration, past history of wheezing and paroxysmal nocturnal cough, urticaria induced by food, and any medication. Among 106 CB donors, complete information was available from 90 neonates (84.9%) after 1-y follow up.

Infants were classified as having atopic disease when a definite diagnosis of atopic dermatitis, food-induced urticaria, and asthma was made by physicians. Atopic dermatitis was diagnosed by the criteria of Hanifin (15). Because the subjects were young infants, the diagnosis of atopic dermatitis was made when an eczematous rash with a typical appearance and distribution of atopic dermatitis existed for 2 mo or longer, as described previously (4, 5). To exclude seborrheic dermatitis as far as possible, the diagnosis of atopic dermatitis was withheld from infants with eczema cured by 3 mo of age. Asthma/recurrent wheezing was diagnosed in infants who had been diagnosed as bronchial asthma by physicians or presented three or more separate episodes of wheezing or paroxysmal nocturnal cough with sleep disturbance for three consecutive nights in the absence of an apparent common cold and other respiratory infections, as described previously (16) with a minor modification. The study was performed with informed consent from the parents and approved by the ethics committees of the Fukui Medical University.

Isolation of CB CD4$^+$ T cells and culture conditions. $CD4^+$ T cells were isolated from CB as described with a minor modification (17). Briefly, mononuclear cells were obtained by centrifugation on Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Tokyo, Japan) and were treated with L-Leucyl methyl ester and Lympho-kwik T helper (One Lambda, Canoga Park, CA, U.S.A.). The resulting population contained >98% viable (trypan blue negative), >98% $CD3^+CD4^+CD8^-$ $CD45RA^+$, and no detectable $CD45RO^{hi}$, $CD25^+$, $CD19^+$, and $CD56^+$ cells.

$CD4^+$ T cells (1×10^6/mL) were cultured in 48-well culture plates in 0.5 mL of RPMI 1640 medium containing 10% FCS, 50 IU/L penicillin G, and 50 μg/L streptomycin and were stimulated with anti-CD3 MAb (200 ng/mL: BD PharMingen, San Diego, CA, U.S.A.) immobilized on mitomycin C-treated CD32/B7–2 L cells (0.25×10^6/mL) in the presence or absence of rhIL-12 (5 ng/mL: PeproTech, Rocky Hill, NJ, U.S.A.). After 72 h, the cell-free supernatants were harvested and stored at −80°C until further analysis. The culture period was determined based on our previous reports in which the levels of IL-13 and IFN-γ reached a plateau 72 h after primary stimulation (18). In some experiments, the stimulated cells were expanded (25 ± 6-fold expansion) with IL-2 (50 U/mL, Shionogi, Osaka, Japan) for 4 d and then restimulated in the same manner. After 24 h of restimulation, the cell-free supernatants were harvested and IL-4 and IFN-γ levels were determined.

Cytokine measurements. IL-13, IL-4, and IFN-γ were measured by ELISA. Antibody pairs and standard recombinant human cytokines for the ELISA were purchased from Endogen Corporation (Woburn, MA, U.S.A.) and PeproTech, respectively. The detection limits for IL-13, IL-4, and IFN-γ were 62.5 pg/mL, 62.5 pg/mL, and 31 pg/mL, respectively.

Statistical analysis. Statistical analyses were performed using the StatView version 5.0 (Abacus Concepts, Berkeley, CA, U.S.A.). The values of samples under the detection limit of ELISA were assigned to a half value of the lowest standard. Mann-Whitney nonparametric *U* tests were used for comparisons between groups as none of cytokine levels was normally distributed. Spearman's rank correlation test was used to determine the relationship of cytokine levels. Statistical significance was inferred if the p value was <0.05. Univariate and multivariate associations between predictor variables and the later development of allergic diseases were tested by logistic regression analysis.

RESULTS

Occurrence of atopic manifestations. Among the 90 infants who participated in the follow-up study, 19 developed one or more episodes of wheezing, and 56 had suffered from eczema by their first birthday. According to the criteria, the diagnosis of atopic disorders was made in 39 out of 90 (43%) of the infants at 1 y of age; 11 had asthma/recurrent wheezing, 32 had atopic dermatitis, and 8 had food-induced urticaria (Table 1). Seven out of 11 infants who received the diagnosis of asthma had other atopic diseases.

Table 1. *Characteristics of study subjects who were followed up for 1 y*

	Infants without atopic disease	Infants with atopic disease
Total (male:female)	51 (30:21)	39 (24:15)
Manifestation (male:female)		
Eczema	22 (10:12)*	34 (22:12)†
Episode of wheezing	3 (1:2)‡	16 (11:5)§
Type of allergic disorders		
Atopic dermatitis	0*	32 (21:11)†
Recurrent wheezing/asthma	0‡	11 (8:3)§
Food-induced urticaria	0	8 (3:5)
Parental history of atopic diseases		
Negative	27	14
Positive (Father:Mother)	24 (13:15)	25 (21:14)
Type of allergic disorders		
Atopic dermatitis	3 (0:3)	6 (0:6)
Asthma	7 (2:5)	7 (4:3)
Allergic rhinitis	19 (11:11)	18 (11:8)

* Although the 22 infants showed eczema, they were not diagnosed as atopic dermatitis because the eczema was diagnosed as other skin diseases, or was cured within 2 mo or by 3 mo of age.

† Two out of 34 infants also manifested asthma or urticaria, and their eczema was cured within 2 mo.

‡ Because the three infants had only one or two episode(s) of wheezing, they were not diagnosed as an asthmatic.

§ Four and one out of 16 infants also manifested atopic dermatitis and urticaria, respectively, and they had only one or two episode(s) of wheezing.

Relationship between atopy and cytokine production by CB CD4$^+$ T cells. We first examined whether the levels of IL-13 and IFN-γ production by CB naive CD4$^+$ T cells at the primary stimulation reflect differentiation ability to Th1/Th2. For this purpose, we measured IL-4 and IFN-γ producing abilities of primed T cells upon restimulation, and compared these levels with IL-13 and IFN-γ production profiles by the same cells at the priming culture. IL-4 levels at the restimulation correlated with IL-13 levels at the primary culture ($r = 0.75$, $p < 0.01$, $n = 20$, Spearman's test), and IFN-γ levels of the primary cultures correlated with those of secondary cultures ($r = 0.98$, $p < 0.005$, $n = 20$, Spearman's test). Thus, we used IL-13 and IFN-γ production at priming as markers to characterize helper T cell differentiation property of neonatal CD4$^+$ T cells.

As depicted in Figure 1*A*, the CB CD4$^+$ T cells from some individuals predominantly produced IFN-γ, indicating that not all neonatal CD4$^+$ T cells skewed to Th2 differentiation. IL-13 and IFN-γ levels showed a negative correlation, suggesting that the differences in cytokine production profile were not an experimental artifact resulting from various factors such as cell viability. Compared with the nonatopic group of infants, the atopic group tended to have more Th2-like cytokine profile with higher IL-13 (Fig. 1*B*), although there was no statistically significant difference in IFN-γ levels between atopic and nonatopic groups (Fig. 1*C*).

IL-12 is a crucial factor for the development of Th1 cells, which may counter-regulate Th2 differentiation (8). As previously reported (19), exogenous IL-12 markedly enhanced IFN-γ production and suppressed IL-13 production (compare Fig. 1*A* and Fig. 2*A*). Although most CB samples predominantly produced IFN-γ in the presence of IL-12, T cells from several individuals still produced high levels of IL-13 (Fig. 2*A*). The IL-13 levels in the presence of exogenous IL-12 were also higher ($p < 0.05$) in atopic group than in nonatopic neonates (Fig. 2*B*), whereas there was no significant difference in the IFN-γ levels (Fig. 2*C*).

Relationship between parental allergic history and cytokine production by CB CD4$^+$ T cells. Forty-nine of 90 (54%) infants had a positive parental history of atopic diseases, among which the incidence of allergic rhinitis was the highest (41%), whereas that of atopic dermatitis was the lowest (10%) (Table 1). The parental history of atopy as a genetic risk factor did not correlate with cytokine production property irrespective of the addition of exogenous IL-12 (data not shown).

Relationship between clinical course of eczema and cytokine production by CB CD4$^+$ T cells. Because chronicity of

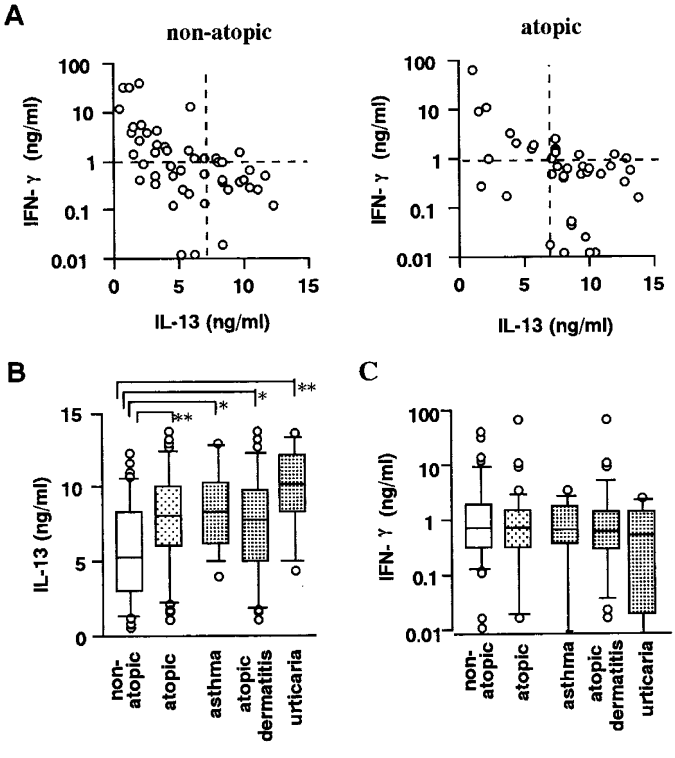

Figure 1. Relationship between cytokine producing ability of CB CD4$^+$ T cells and subsequent development of atopic diseases. CB CD4$^+$ T cells were stimulated with anti-CD3 MAb and B7–2. Cytokine production profiles of nonatopic (*left panel*) and atopic (*right panel*) infants were plotted (*A*). Vertical and horizontal broken lines correspond to 7 ng/mL of IL-13 and 1 ng/mL of IFN-γ, respectively, which were determined by receiver operating characteristic curves. IL-13 (*B*) and IFN-γ (*C*) production by CB CD4$^+$ T cells from infants with various atopic diseases are shown as 10th, 25th, 50th, 75th, and 90th percentiles, and comparisons were made by Mann-Whitney *U* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

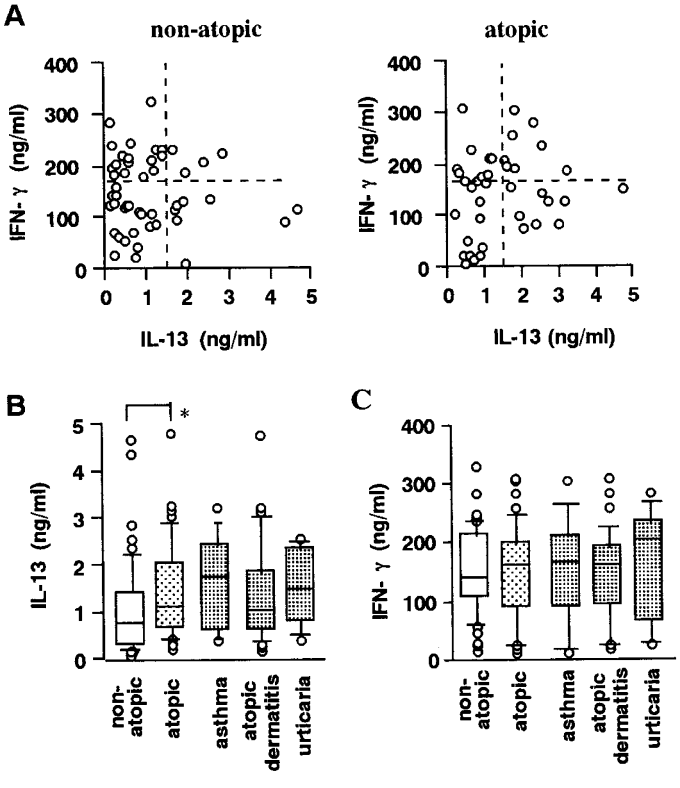

Figure 2. Effects of exogenous IL-12 on the correlation between cytokine producing ability of CB CD4$^+$ T cells and subsequent development of atopic diseases. CB CD4$^+$ T cells were stimulated with anti-CD3 MAb and B7–2 in the presence of IL-12. Cytokine production profiles of nonatopic (*left panel*) and atopic (*right panel*) infants were plotted (*A*). Vertical and horizontal broken lines correspond to 1.5 ng/mL of IL-13 and 170 ng/mL of IFN-γ, respectively, which were determined by receiver operating characteristic curves. IL-13 (*B*) and IFN-γ (*C*) production by CB CD4$^+$ T cells from infants with various atopic diseases are shown as 10th, 25th, 50th, 75th, and 90th percentiles, and comparisons were made by Mann-Whitney *U* test. *$p < 0.05$.

eczema is a characteristic feature of atopic dermatitis, we next analyzed the correlation between the onset and duration of eczema and cytokine production pattern. As shown in Figure 3, CB $CD4^+$ T cells of infants with eczema for 2 mo or longer produced higher levels of IL-13 in the presence or absence of IL-12 than those with transient eczema. Higher IFN-γ production in the presence of IL-12 was also observed in neonates with chronic eczema than in those with short-term eczema, but this was not the case in the absence of IL-12. The age of onset did not correlate with either IL-13 or IFN-γ levels (data not shown).

Cytokine levels as predictors of atopic diseases. Finally, we evaluated the usefulness of cytokine levels for the prediction of later development of atopic disease. First, the following variables were analyzed as univariate predictors of allergic diseases: IL-13 and IFN-γ production by CB $CD4^+$ T cells in the presence or absence of IL-12, gender, and parental history of allergic diseases. The IL-13 production in the absence of IL-12 was the most highly related to the development of atopic disease [$p = 0.005$, odds ratio (OR) 1.22], followed by the IL-13 production in the presence of IL-12 and parental history, neither of which, however, were statistically significant (Table 2).

Because the levels of IL-13 and IFN-γ were not always independent variables as shown in Figure 1, these parameters were also analyzed according to the multivariate models adjusting for interaction between cytokines. The IL-13 production in the absence of IL-12 was also the most highly related to atopic diseases ($p = 0.009$, OR 1.30), but not in the presence of IL-12 ($p = 0.799$). Finally, using a stepwise regression method, IL-13 production in the absence of IL-12 ($p = 0.003$, OR 1.24) and parental history of allergic diseases ($p = 0.042$, OR 2.64) were selected as predictive risk factors. IFN-γ levels did not seem to be useful for the prediction of atopic disease in any analyses.

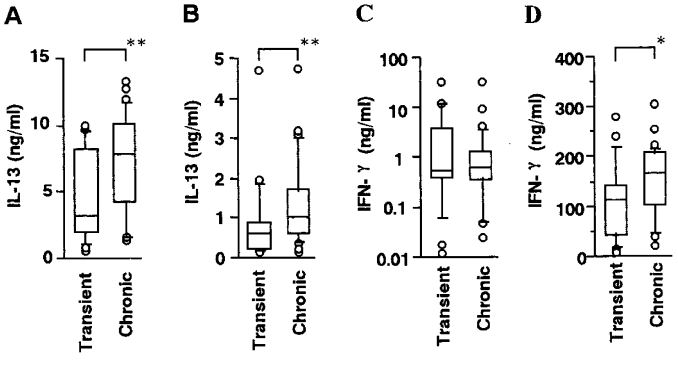

Figure 3. Relationship between production of IL-13 and IFN-γ production by CB $CD4^+$ T cells and the duration of eczematous skin lesion. CB $CD4^+$ T cells were stimulated with anti-CD3 MAb and B7–2 in the absence (*A* and *C*) or presence (*B* and *D*) of IL-12. IL-13 (*A* and *B*) and IFN-γ (*C* and *D*) production by CB $CD4^+$ T cells from infants with eczema for 2 mo or longer (chronic) and those with short-term eczema (transient) are shown as 10th, 25th, 50th, 75th, and 90th percentiles, and comparisons were made by Mann-Whitney *U* test. $*p < 0.05$; $**p < 0.02$.

DISCUSSION

In this study, we examined whether the intrinsic cytokine-producing property of CB naive $CD4^+$ T cells is associated with the later development of atopic diseases in a cohort of infants. We found that not all cytokine production profiles of CB $CD4^+$ T cells showed skewed Th2 patterns and that infants who subsequently develop atopic disease more often have CB $CD4^+$ T cells that produce amounts of IL-13 in the higher ranges. The high IL-13 producing ability of CB $CD4^+$ T cells seems to be a useful marker in newborns for the prediction of later development of atopic diseases. Previous studies have anticipated that reduced levels of IFN-γ production by T cells of high-risk neonates was likely to be associated with increased production of Th2-type cytokines such as IL-13 (7–11). Increased IL-13 production by peripheral blood mononuclear cells of patients with established atopic disease has been shown (20). Our study is the first report that clearly demonstrated the high IL-13 producing ability of naive $CD4^+$ T cells of high-risk neonates before manifestation of atopic symptoms.

After 1-y follow-up, we observed a high prevalence of atopic disease manifestation compared with other reports (21). A prospective study in Japan (22), however, demonstrated that infants born in winter showed the highest cumulative incidence (33.9%) of atopic dermatitis compared with those born in other seasons. Mar *et al.* (23) reported that the 12-mo cumulative incidence of atopic dermatitis in Chinese infants born in Melbourne was 44%, whereas that in Caucasians was only 21%. Thus, the high prevalence might be explained by the birth season of subjects and/or genetic differences as well as the criteria used in the different studies.

In contrast to our results, Williams *et al.* (16) reported that PHA-stimulated mononuclear cells of neonates with either a parental history of atopy or manifestation of allergic symptoms produced lower amounts of IL-13 compared with the control. Moreover, Prescott *et al.* (24) demonstrated that the allergen-specific production of Th2 cytokines including IL-13 as well as that of IFN-γ is significantly lower in CB mononuclear cells of the neonates who develop atopy. Several explanations can be invoked to explain these controversial findings. First, because they did not use highly purified $CD4^+$ T cells, secretion of inhibitory cytokines, such as IL-12 and/or consumption by other bystander cells than $CD4^+$ T cells may affect IL-13 levels. Second, the proportion of the antigen-specific T cells is assumed to be extremely low in CB, and polyclonally stimulated naive T cells produce IL-13 as well as memory T cells (14). Thus the IL-13 levels in this study presumably reflect the intrinsic property of naive T cells rather than that of memory ones, whereas allergen-specific IL-13 production may reflects memory T cells primed antenatally, as addressed by Prescott (24). Third, antigen-specific IL-6 production by non-T cell population of CB was different between atopic and nonatopic groups (7). Given that antigen-specific stimulation depends on interaction between T cells and APC, immaturity and dysregulation of APC in atopic group may be involved in the lower IL-13 production, although no conclusive evidence is present (25). Finally, the nonatopic group in this study may include atopic infants who did not manifest allergic symptom by 1 y of

Table 2. *Factors associated with subsequent development of atopic diseases*

	Univariate analysis			Multivariate analysis		
	Unadjusted OR	95% CI	*p* Value	Adjusted OR	95% CI	*p* Value
The initial model						
IL-13 level without IL-12	**1.22**	1.06–1.39	**0.0045**	**1.30**	1.07–1.58	**0.009**
IL-13 level with IL-12	1.42	0.93–2.21	0.108	1.08	0.59–1.97	0.799
IFN-γ level without IL-12	0.99	0.94–1.04	0.685	1.05	0.99–1.11	0.130
IFN-γ level with IL-12	1.00	0.99–1.01	0.854	1.00	0.99–1.00	0.291
Positive parental history	2.01	0.86–4.72	0.110	**3.29**	1.19–9.12	**0.022**
Gender, male	1.12	0.48–2.63	0.795	1.03	0.38–2.77	0.961
The final model						
IL-13 level without IL-12				**1.24**	1.08–1.43	**0.003**
Positive parental family history				**2.64**	1.04–6.73	**0.042**

Unadjusted and adjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated by logistic regression analysis taking into account all other risk factors of this table that were obtainable at birth. The final model was determined by a stepwise regression method. Bold numbers mean statistically significant.

age, and the atopic group included infants with mild symptom. The different criteria for the classification might explain the discrepancy.

Although several studies have demonstrated that reduced IFN-γ production by CB mononuclear cells is a marker of high risk for allergy (3–7), we could not detect a significant correlation between IFN-γ levels and subsequent development of allergic diseases. As Liao *et al.* (7) addressed, the low number of natural killer cells and the altered monocyte function of high-risk neonates may account for the decreased IFN-γ production by CB mononuclear cells. It is well established that IFN-γ derived from T cells and natural killer cells enhances IL-12 production by monocytes and dendritic cells, and IL-12 augments IFN-γ production by T cells (26). This positive feedback loop seems to manifest a difference in IFN-γ producing ability of CB mononuclear cells. In any case, the total understanding of the immunologic basis for the skewed Th cytokine producing profiles in atopic children needs further investigations.

Even in the presence of exogenous IL-12, a higher IL-13 production by CB $CD4^+$ T cells was noted in the atopic group than in the nonatopic one. Given that microbial agents are a potent stimulator of IL-12 production, $CD4^+$ T cells from atopy-prone neonates would be able to produce IL-13 upon microbial exposure, resulting in amelioration of allergic inflammation. It has been shown that acute T cell infiltration in atopic dermatitis is associated with a predominance of IL-4 and IL-13 expression, whereas maintenance of chronic inflammation is associated with increased IL-12 and IFN-γ expression (12, 27). It is intriguing that in the both presence and absence of IL-12, $CD4^+$ T cells of neonates who later developed eczema for longer periods produced higher levels of IL-13 than those with shorter periods of eczema. Moreover, higher IFN-γ production responding to exogenous IL-12 was observed in neonates with chronic eczema than those with short-term eczema. Thus, the IL-13 producing ability and IL-12-induced IFN-γ production of $CD4^+$ T cells seems to play an important role in the pathogenesis of atopic dermatitis.

The family history was not related to cytokine producing ability of neonatal $CD4^+$ T cells. We included history of allergic rhinitis against pollen, which has recently increased among Japanese population and is usually seen in adolescents and adults. The genetic and environmental background of pollen allergy may be different from that of infantile atopy. Moreover, parents may forget their own history of atopic diseases in childhood. Thus, the significance of parental history of atopic diseases as a genetic background involved in Th1/Th2 differentiation is dependent on several uncontrollable factors.

IL-13 gene polymorphism has been recently reported to be associated with a high total serum IgE level and increased risk of atopic asthma and atopic dermatitis (28, 29). If the IL-13 producing ability of neonatal $CD4^+$ T cells correlates with polymorphisms of IL-13 gene, it would be an important evidence for the predictive value of polymorphism of IL-13 gene. Moreover, it should also provide a better understanding of the regulation of IL-13 production and its role in the pathogenesis of atopic disease.

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