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Cytokine profiles in 1-yr-old very low-birth-weight infants after enteral glutamine supplementation in the neonatal period

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In a previous study, we found that glutamine-enriched enteral nutrition in 102 very low-birth-weight (VLBW) infants decreased both the incidence of serious neonatal infections and atopic dermatitis during the first year of life. The aims of this follow-up study were to determine whether these beneficial effects are attended by changes in Th_1 and Th_2 cytokine profiles at age 1 yr. Furthermore, we studied changes in cytokine profiles during the first year of life in these VLBW infants. In total, 89 infants were eligible for the follow-up study (12 died, 1 exclusion due to a chromosomal abnormality). Th_1 (IFN-γ, TNF-α and IL-2) and Th_2 cytokine (IL-10, IL-5, and IL-4) profiles following *in vitro* whole blood stimulation were measured at 1 yr. Cytokine profiles were measured in 59/89 (66%) infants. Glutamine-enriched enteral nutrition in neonatal period did not influence cytokine profiles at 1 yr. Cytokine profiles were not different in infants with and without allergic or infectious diseases. The beneficial effect of glutamine-enriched enteral nutrition on the incidence of serious neonatal infections and atopic dermatitis during the first year of life is not related to changes in the Th_1 and Th_2 cytokine profiles. Both Th_1 and Th_2 cytokine profiles increased during the first year of life in this cohort of VLBW infants.

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Key words: infant; very low-birth-weight; glutamine; follow-up study; Th_1/Th_2 cytokines

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In a previous study, we found that glutamine-enriched enteral nutrition in the neonatal period decreased both the incidence of serious neonatal infections and atopic dermatitis during the first year of life in very low-birth-weight (VLBW) infants (1, 2). However, in other studies of both enteral (3) and parenteral (4) glutamine supplementation, conflicting results were found regarding the incidence of neonatal infections. The mechanism underlying the potential beneficial effect of glutamine supplementation is not fully understood, but may include the enhancement of the systemic immune response.

In a murine asthma model, Ko et al. (5) found that systemic glutamine administration suppressed the key features of asthma, induced by T-helper type 2 cells, by suppression of the production of Th_2 cytokines (IL-4, IL-5 and IL-13). As such, we hypothesized that the lower rate of atopic dermatitis in VLBW infants receiving glutamine-enriched enteral nutrition in the neonatal period is the result of similar immune mechanisms. Therefore, we determined Th_1 and Th_2 cytokine profiles at the age of 1 yr in relation to:

(i) neonatal glutamine supplementation;
(ii) serious neonatal infections; and
(iii) atopic dermatitis during the first year of life.

Furthermore, we assessed the changes in cytokine profiles during the first year of life in VLBW infants.

Methods

The initial study was a randomized, controlled trial of glutamine-enriched enteral nutrition in 102 VLBW infants, (2) as previously described in the study protocol (6). Baseline characteristics of the follow-up cohort and their mothers were recorded in the initial study (2). Data on clinical outcome, including allergic and infectious diseases, during the first year of life were previously published (1).

At age 1 yr, 89 VLBW infants were eligible to participate in the follow-up study (12 died, 1 excluded due to a chromosomal abnormality). The national central committee on research involving human subject and the medical ethical review board of our institute approved the study protocol. All parents gave informed written consent.

At age 1 yr, 1 ml of capillary blood was obtained and stimulated in the presence of anti-CD3/anti-CD28 and *Eschericia coli* lipopolysaccharide (concentration 1:1000 both). IFN-γ, TNF-α, IL-10, IL-5 and IL-2 were determined by cytometric bead array (CBA) (BD Biosciences, Alphen aan den Rijn, the Netherlands). In cytometric bead array, six bead populations with distinct fluorescence intensities are coated with specific antibodies to capture different cytokines in plasma. The six cytokine-captured beads were mixed into a solution. 5 μl of the mixed beads solution was mixed with 5 μl phycoerythrin-conjugated detection antibodies and 5 μl recombinant standards or test samples to form sandwich complexes. IL-4 was measured by ELISA (PeliKine, Sanquin Reagents, Amsterdam, the Netherlands) (7).

Baseline characteristics were analyzed by Student's *t*-test (for normally distributed continuous data), Mann–Whitney *U*-test (for non-parametric continuous data), chi-square test or Fisher's exact test (for dichotomous data). Generalized estimating equations were used to analyze changes in cytokine responses over time. Regression analysis was used to determine the effect of antenatal corticosteroids, chorioamnionitis, mode of delivery, gestational age, birth weight and serious neonatal infection on cytokine responses at age 1 yr $p < 0.05$ were considered significant. SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses.

Results

Between December 2002 and October 2004, 59/89 (66%) infants participated. Baseline maternal and infant characteristics were not different between the participating (n = 59) and non-participating (n = 30) group (data not shown). Baseline infant and maternal characteristics were not different between glutamine-supplemented (n = 28) and control group (n = 31) (Table 1).

At 1 yr of age, Th_1 (IFN-γ, TNF-α and IL-2) and Th_2 (IL-5. IL-4 and IL-10) cytokine profiles were not different between glutamine-supplemented and control groups (Table 2).

Information about atopic dermatitis and bronchial hyper-reactivity of 52/59 (88%) infants during the first year of life was available. Cytokine profiles at 1 yr of age were not different between infants with (n = 8) and without (n = 44) atopic dermatitis during the first year of life, nor between infants with (n = 7) and without (n = 45) bronchial hyper-reactivity (data not shown). Similarly, cytokine profiles were not different between infants with (n = 36) and without (n = 23) serious neonatal infection (data not shown).

Blood samples were taken in neonatal period at 2 ($t = 0$), 7 ($t = 1$), 14 ($t = 2$) days after birth and at 1 yr ($t = 3$). Following incubation with anti (α)-CD3/ α-CD28, IFN-γ ($p < 0.001$),

Table 1. Baseline characteristics

	Glutamine (n = 28)	Control (n = 31)	p*
Maternal age at birth (yr)	29.7 ± 5.1	29.3 ± 5.8	0.77
Race (% Caucasian)	23/28 (82%)	25/31 (81%)	0.88
High maternal education†	10/28 (36%)	9/31 (29%)	0.58
Antenatal corticosteroids	20/28 (71%)	27/31 (87%)	0.15
Vaginal delivery (%)	12/28 (43%)	11/31 (35%)	0.56
Gestational age (wk)	29.4 ± 1.7	28.9 ± 2.0	0.22
Birth weight (kg)	1.27 (0.4)	1.19 (0.3)	0.35
Birth weight <p10‡ (%)	6/28 (21%)	8/31 (26%)	0.69
Sex (% male)	14/28 (50%)	16/31 (52%)	0.90
5-Minute Apgar score ≤ 6	2/28 (7%)	2/31 (6%)	0.91
Administration of surfactant	9/28 (32%)	17/31 (55%)	0.12
Postnatal corticosteroids§	1/28 (4%)	3/31 (10%)	0.35
≥1 serious infection in the neonatal period¶	14/28 (50%)	22/31 (71%)	0.09
Age at discharge from NICU (d)	22 (3–104)	18 (2–91)	0.09
Age at discharge from hospital (d)	63 (35–162)	56 (35–129)	0.17
Age at follow-up (yr)	1.3 ± 0.2	1.2 ± 0.1	0.22
Age at follow-up –1 yr of corrected age (wk)	0.8 (–4.9–12.0)	0.9 (–5.1–20.8)	0.63

Data are means ± s.d., medians (ranges) or number (%).
*Student's *t*-test, Mann–Whitney *U*-test and chi-square or Fisher's exact test were used to analyze continuous normally distributed, non-parametric continuous and dichotomous data, respectively.
†Higher professional or university education.
‡According to Usher.
§More than 11 days.
¶Sepsis, meningitis, pyelonephritis, pneumonia or arthritis as diagnosed by a combination of clinical signs and a positive culture.

Table 2. Cytokine profiles at 1 yr of age

	Glutamine (n = 28)		Control (n = 31)		
	Median (pg/ml)	25th–75th percentile (pg/ml)	Median (pg/ml)	25th–75th percentile (pg/ml)	p*
LPS					
IFN-γ†	259	69–723	216	49–1091	0.77
TNF-α†	1122	380–1769	1030	220–2552	0.75
IL-10†	1733	1128–2768	2230	1395–2953	0.29
IL-5‡					
IL-4§	3.9	2.6–8.8	2.6	2.6–4.9	0.14
IL-2‡					
Anti (α) CD3/αCD28					
IFN-γ†	2620	661–4120	1413	716–3333	0.45
TNF-α†	289	96–621	126	50–550	0.24
IL-10†	713	260–1667	384	198–1099	0.21
IL-5†	242	107–503	223	91–412	0.53
IL-4§	11.4	6.4–35.8	9.1	3.5–19.5	0.15
IL-2†	1417	235–3303	385	178–1255	0.09

*Mann–Whitney *U*-test.
†Analyzed by Cytometric Bead Array (CBA).
‡For IL-5 and IL-2 only levels following incubation with anti(α)-CD3/α-CD28 are presented, as following incubation with LPS IL-5 and IL-2 levels were below detection limit (40 pg/ml) in more than 85% of the cases.
§Analyzed by enzyme-linked immunosorbent assay (ELISA).

TNF-α ($p = 0.010$), IL-10 ($p < 0.001$), IL-4 ($p < 0.001$) and IL-5 ($p < 0.001$) levels increased between neonatal period and at 1 yr of age, irrespective of the neonatal treatment. Following incubation with LPS, IFN-γ ($p < 0.001$). IL-4 ($p < 0.001$) and IL-10 ($p < 0.001$) levels increased between start of the study and 1 yr (data not shown).

Discussion

In our study in VLBW infants, we found that the beneficial effect of glutamine-enriched enteral nutrition on atopic dermatitis during the first year of life is not related to changes in Th_1 and Th_2 cytokine profiles. The development of the T-cell function during childhood has been previously studied. Atopic diseases are associated with an overt Th_2 cytokine profile (8). Early exposure to microbes will induce Th_1 cytokine responses that shift presumed neonatal default Th2 immune responses towards a balanced Th_1/Th_2 cytokine profile (9). Consequently, delayed transition into adult Th_1-polarized cytokine responses may lead to long-term persistent Th_2 responses and subsequent allergic disease (10). In high-risk infants, van der Velden et al. (11) found that the first six months of life may represent a critical phase for the induction of atopy associated immune responses in later life. As such, an increased production of Th_2 cytokines was found, possibly due to the absence of counter-regulatory events, e.g. induction of IFN-γ producing Th_1 cells in the neonatal period (11).

Kalliomaki et al. (12) found that these immunological events can be modulated by probiotics. We hypothesized that the lower incidence of serious neonatal infections in VLBW infants may result from intervention with glutamine supplementation and is reflected by enhanced Th_1 cytokine responses. However, we found no differences in Th_1 and Th_2 cytokine levels between atopic and non-atopic infants, at 1 yr of age. Comparison between our study and the earlier mentioned studies (11, 12) is hampered due to differences in study population and methods of determination of cytokine profiles.

In our previous study (7), we found no effect of glutamine-enriched enteral nutrition in VLBW infants in the neonatal period on cytokine profiles in neonatal period. In the current study, we specifically addressed whether glutamine administration would positively affect the immunological process, characterized by a physiological switch from a Th_2 cytokine profile in the neonatal period into a modified Th_2 profile later in life. Similar to our previous neonatal analyses, we found ample cytokine responses by peripheral immune-cells with large inter-individual differences. No differences in Th_1 and Th_2 cytokine profiles were observed between glutamine supplemented and control infants.

So far, little information is available on the development of the Th_1 and Th_2 cytokine profiles during the first year of life of VLBW infants. In our study, both Th_1 (IFN-γ, TNF-α) and Th_2 (IL-10, IL-4) cytokines increased during the first year of life, irrespective of the neonatal treatment. This is in line with the increase of memory cells during the first year of life.

One aspect of our study design needs to be addressed. Our sample size for the follow-up study, based on the sample size calculation for the primary outcome of the initial trial, was relatively small. Therefore, the results of our study may be susceptible to a type II error.

In conclusion, this is the first study into the effects of neonatal glutamine-enriched enteral nutrition in VLBW infants on Th_1 and Th_2 cytokine profiles at the age of 1 yr. The beneficial effect of glutamine-enriched enteral nutrition on the incidence of serious neonatal infections and atopic dermatitis during the first year of life is not related to changes in the immune responses, reflected by Th_1 and Th_2 cytokine profiles. Both Th_1 and Th_2 cytokine profiles increased between the neonatal period and 1 yr in VLBW infants.

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