

Maternally delivered nutritive allergens in cord blood and in placental tissue of term and preterm neonates

M. Edelbauer, C. Loibichler, I. Nentwich, M. Gerstmayr, R. Urbanek and Z. Szépfalusi

Department of Paediatrics, University of Vienna, Austria

Summary

Background The proliferation of cord blood mononuclear cells in response to nutritive and inhalant allergens implies intrauterine exposure with resulting T cell priming. However, the mechanisms triggering these fetal allergen-specific immune responses are incompletely understood.

Methods We studied the placental release of endogenous β -lactoglobulin (BLG) and ovalbumin (OVA) by the use of an open *ex vivo* placental perfusion model. Preterm and term placentas were obtained immediately after delivery to recover functionally active fetal and maternal circulations. Fetal and maternal perfusate samples were collected throughout the perfusion experiments with medium. Matched cord blood samples were collected separately. All samples were tested for the presence of OVA and BLG by allergen-specific ELISAs.

Results In 16 out of 19 placentas, the nutritive allergens could be detected both in fetal and maternal perfusate samples. Fetal wash out levels of the allergens BLG and OVA from the placental tissue of preterm and term deliveries were observed in traces and up to 44.4 and 2.6 ng/mL, respectively. In cord blood of preterm and term neonates, BLG and OVA could be detected at concentrations up to 16.7 and 5 ng/mL, respectively.

Conclusion These findings provide direct evidence for the release of tiny amounts of nutritive allergens from placental tissue indicating diaplacental allergen transfer and fetal exposure to nutritive allergens *in vivo*.

Keywords: BLG, cord blood, OVA, placenta

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Introduction

Allergen-specific T cell responses at birth indicate intrauterine exposure of the fetus to antigens and priming of the fetal immune system [1–5]. Two routes of allergen transfer to the fetus have been postulated, the dia-amniotic [6] and the diaplacental transport [7]. Exposure to allergens early in life may have an impact on the incidence of allergy many years later. Thus, factors that protect or enhance the development of atopic diseases appear to be acting early in life. In this respect, the gestational environment is of importance, since allergen-specific immunity has been demonstrated to develop *in utero* [2], and maternal atopy was found to be a stronger risk factor for the infant to develop atopic disease than paternal atopy [8]. However, the proliferative responses of cord blood mononuclear cells (CBMCs) to allergens have been shown to be not directly related to maternal allergen exposure and not to predict the allergic outcome of the infant [9, 10].

Recently, we could demonstrate that diaplacental transport of ovalbumin (OVA) and β -lactoglobulin (BLG) *in vitro* occurs in a dose-dependent and molecular-weight-dependent

manner [11]. Thus, the objective of this work was to investigate (a) placental tissue and placental vessels for the presence of maternally ingested nutritive allergens, which can be washed out by perfusing the placenta and (b) whether maternal milk and egg consumption relates to allergen concentration in fetal circulation.

Materials and methods

Subjects

Twenty-seven healthy pregnant women at term (>36 weeks of gestation) as well as preterm mothers (27–36 weeks of gestation) were recruited for the study. This was an unselected group of consecutive presentations to the General Hospital of Vienna, Austria. All pregnant women were not diet restricted. Data on ingestion, including frequency and quantity of cows' milk, hens' eggs and milk- and egg-containing nutrimentals such as noodles or cake in the last week before parturition were collected by a standardized maternal questionnaire and maternal interview just prior to the delivery of their infants.

Placentas and cord blood

Placentas of preterm ($n = 9$) and term neonates ($n = 18$) were obtained immediately after vaginal delivery or caesarean

Correspondence: Zsolt Szépfalusi, MD, Clinic of Paediatrics, University of Vienna Medical School, Währinger Gürtel 18–20, A-1090 Vienna, Austria.
E-mail: zsolt.szepfalusi@akh-wien.ac.at

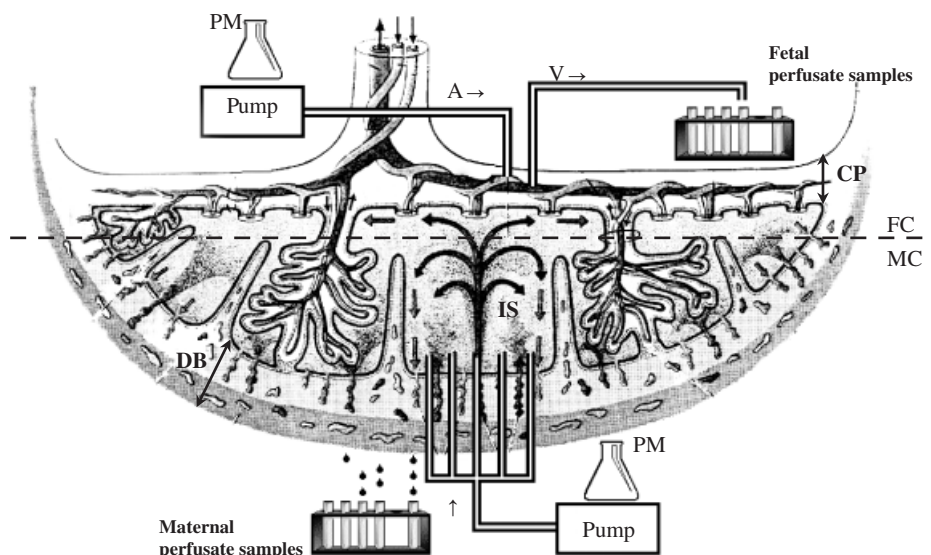


Fig. 1. Schematic diagram of the placental perfusion system. *Fetal circulation (FC)*: After cannulation of an artery (A) and the corresponding vein (V) at the junction to the chorionic plate (CP), fetal vessels were rinsed with perfusion medium (PM). *Maternal circulation (MC)*: Five cannulas were placed in the intervillous space (IS) and were flushed with perfusion medium (PM). Samples of fetal venous outflow (= fetal perfusate samples) and reflux from intervillous space (= maternal perfusate samples) through veins in the decidua basalis (DB) were collected throughout the perfusion experiment. Arrows indicate flow direction.

sections. Matched cord blood was collected by puncture of the placental side of the umbilical vein and umbilical artery to separate the serum. Eight placentas were rejected showing tissue leakage, and thus were not included in the number of preterm and term placentas used in this study.

Open *ex vivo* perfusion experiments of the whole placenta have been established with modifications from the original technique [12–14]. Briefly, the placentas were obtained immediately after delivery to recover active feto- and materno-placental circulations by cannulating an artery and a vein on the chorionic plate and flushing the intervillous space by piercing five cannulas through the decidua basalis. Reflux of maternal blood from the intervillous space passing through veins in the decidua basalis was collected (= maternal perfusate samples). Samples from fetal outflow of the cannulated cord vein (= fetal perfusate samples) were collected separately (Fig. 1). The perfusion medium contained 11 g/L Earl's Medium 199 (Biochrom KG, Berlin, Germany), supplemented with 3 g/L bovine albumin (Behringerwerk AG, Marburg, Germany), 20 g/L dextran T40 (Pharmacia, Uppsala, Sweden) and 10 mL/L sodium hydrogen carbonate (8.4%). pH was adjusted to 7.38–7.42. The medium was warmed to 37 °C and aerated with 95% oxygen and 5% carbon dioxide. Fetal and maternal samples were collected in 1-min intervals up to 10 min.

Measurement of OVA and BLG

Sandwich-ELISAs for the detection of BLG and OVA in perfusate samples and serum have been established in our laboratory. All samples were tested in triplicate. A maximum intra-assay variation of 7% was accepted. Allergen concentrations were determined by extrapolation from the respective standard curve. Unless mentioned otherwise, antibodies and chemicals were purchased from Sigma Chem., CO, USA.

BLG-ELISA Plates were coated with polyclonal affinity purified rabbit-anti-BLG (1:100 in carbonate buffer). The blocking step with human serum albumin (1% in carbonate buffer) was followed by an overnight incubation with samples, standards and wells with buffer without added serum or eluates (blanks). Immune complexes were captured with rabbit-anti-BLG labelled with alkaline phosphatase (1:500 in sodium phosphate buffer). *p*-Nitrophenyl phosphate (1 mg/mL in diethanolamine buffer) was used as a substrate. Both Abs were delivered from Bethyl Lab. Inc., Montgomery, USA. A concentration linearity for BLG standard was found between 0.2 and 100 ng/mL.

OVA-ELISA After the coating of microplates with polyclonal goat-anti-chicken egg albumin (1:1000 in phosphate-buffered saline), and washing with Tris-buffered saline, unspecific bindings were blocked with bovine serum albumin (1% in phosphate-buffered saline). Following an overnight incubation with samples and standards, the allergen-antibody complexes were captured by rabbit-anti-chicken egg albumin (1:5000 in phosphate-buffered saline including 0.5% bovine serum albumin). Both Abs are Cappel Abs from ICN Pharmaceuticals Inc., Costa Mesa, CA, USA. Swine-anti-rabbit labelled with alkaline phosphatase (1:1000 in phosphate-buffered saline including 0.5% bovine serum albumin) was used as detecting Ab. Absorbance was measured at 405 nm after colorimetric reactions with *p*-nitrophenyl phosphate. The sensitivity of the assay was 25 pg/mL.

Statistical analysis

Statistical comparison was made using the Mann-Whitney *U*-test. A *P*-value of less than 0.05 was considered significant.

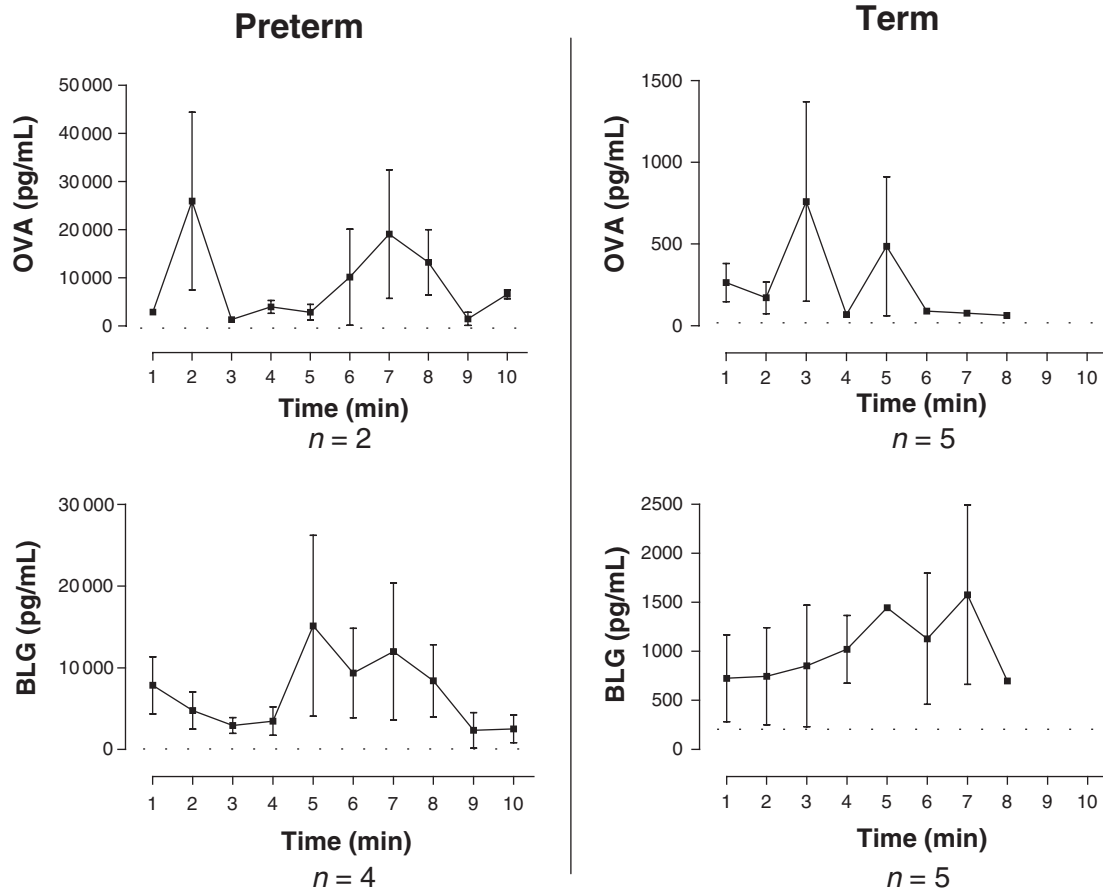


Fig. 2. Release of endogenous ovalbumin (OVA) and β -lactoglobulin (BLG) from placental tissue of term and preterm neonates collected on the fetal side (= fetal perfusate samples). Dotted lines indicate the limit of the detection system. Shown values are mean $\pm$ standard error of the mean (SEM).

Results

Allergen levels in perfusate samples of term and preterm neonates

Fetal perfusate samples The nutritive allergens OVA and BLG were readily detectable in 6 (+2 at the limit of detection) out of 10 and 3 (+2 at the limit of detection) out of eight term placentas, respectively (Fig. 2). BLG and OVA were detected at a mean level of 1.0 ± 0.1 and 0.2 ± 0.1 ng/mL, respectively. The mean BLG and OVA amounts in all preterm placentas ($n = 9$) were 6.9 ± 1.4 and 8.7 ± 2.6 ng/mL. Allergen concentrations measured in all examined preterm placentas were considerably higher than concentrations measured in term placentas ($P = 0.0008$).

Maternal perfusate samples The mean OVA and BLG concentrations measured in all medium perfused preterm placentas were 27.9 ± 10.0 and 13.4 ± 5.9 ng/mL, respectively. In eight out of 10 term placentas, the mean maternal OVA and in five out of eight BLG concentrations in maternal perfusates were 8.1 ± 4.3 and 4.4 ± 1.0 ng/mL.

Allergen concentration in cord blood OVA and BLG were detected in venous and/or arterial cord blood of all premature neonates at a concentration up to 16.7 and 13.0 ng/mL, respectively. The mean allergen levels in cord blood were

4.7 ± 1.7 in arterial and 7.3 ± 1.5 ng/mL in venous samples of all preterm placentas. In cord blood of mature neonates, OVA and BLG were found in nine (+4 at the limit of detection) out of 18 venous and two (six at the limit of detection) out of nine arterial samples up to 5 and 2.9 ng/mL, respectively (Fig. 3). The mean venous and arterial concentrations were 0.75 ± 0.40 and 0.6 ± 0.5 ng/mL, respectively. OVA was detectable in two out of three and BLG was detectable in six out of six cord blood samples from preterm placentas. The overall higher concentration of allergens in placentas of preterm compared with term deliveries could be observed both in cord blood and in fetal perfusate samples. Allergen levels in cord blood and perfusate samples are summarized in Table 1. No correlation was found between the allergen levels detected in venous and arterial samples.

Maternal ingestion and allergens in cord blood A correlation was found between maternal consumption of milk and eggs and the levels of BLG and OVA (Table 2) measured in arterial and venous cord blood. BLG and OVA were even detectable in cord blood with the last maternal uptake of milk and eggs 2–3 days before delivery. BLG or OVA was undetectable or at the limit of detection if the pregnant mothers consumed only milk and egg-containing nutriment.

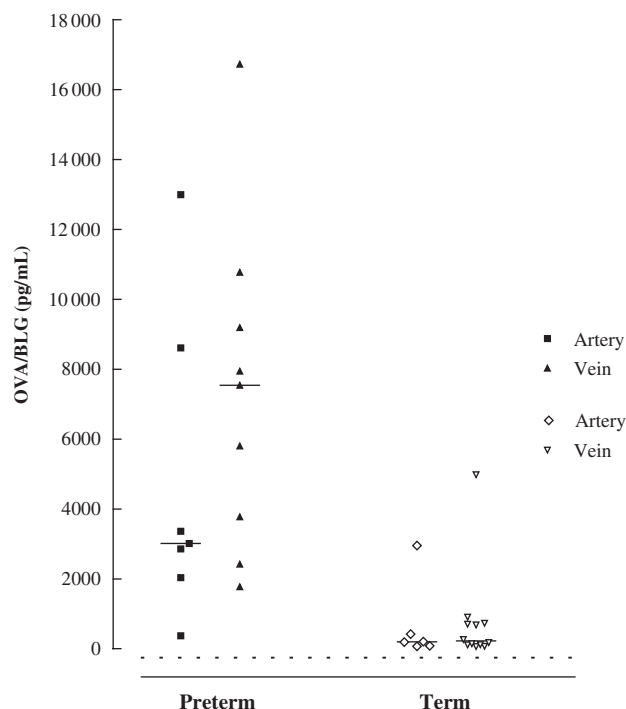


Fig. 3. Ovalbumin (OVA) and β -lactoglobulin (BLG) in arterial and venous cord blood of preterm and term neonates. Dotted line indicates the limit of the detection system.

Table 1. Concentrations (ng/mL) of the nutritive allergens OVA and BLG in perfusate samples and cord blood as determined by allergen-specific ELISA

	Preterm		Term	
	Mean $\pm$ SEM	Highest conc.	Mean $\pm$ SEM	Highest conc.
<i>Fetal perfusate samples</i>				
OVA	8.75 $\pm$ 2.65	44.43	0.25 $\pm$ 0.09	2.59
BLG	6.87 $\pm$ 1.40	37.28	1.03 $\pm$ 0.12	2.49
<i>Cord blood</i>				
Venous	7.33 $\pm$ 1.55	16.73	0.75 $\pm$ 0.40	4.98
Arterial	4.75 $\pm$ 1.68	13.00	0.65 $\pm$ 0.46	2.95

OVA, ovalbumin; BLG, β -lactoglobulin; SEM, standard error of the mean; conc., concentration.

Discussion

As previously observed, nutritive allergens cross the human placenta in a dose-dependent and preterm accentuated manner [11] as shown in a human *ex vivo* placenta model. However, a direct evidence for transplacental allergen transport *in vivo* has not been demonstrated. The results of this study demonstrate that OVA and BLG are detectable in tiny amounts in fetal and maternal perfusate samples and cord blood, thus supporting the idea of prenatal fetal allergen exposure.

There have been several studies showing the reactivity of CBMCs in response to nutritive and inhalant allergens, implying intrauterine allergen exposure with resulting T cell priming [3–5, 15, 16]. Moreover, there is evidence for an

Table 2. Maternal ingestion of cows' milk and hens' eggs and related BLG and OVA levels in cord blood of term and preterm neonates (ng/mL)

	Preterm		Term	
Maternal ingestion	Artery	Vein	Artery	Vein
BLG				
50 mL/day	0.376	1.777		
	2.859	3.772	*	*
< 70 mL/day	–	6.800		
70 mL/day	3.364	7.542	**	0.705
	3.012	7.943	**	0.686
			0.421	0.731
500 mL/day	12.995	16.727		
OVA				
< 1/week			–	*
1/week	1.034	2.419	*	0.143
			–	0.268
			–	0.111
2/week	8.609	9.191	2.953	3.977
	–	10.770	*	0.119
			*	0.909

Data on ingestion of milk and eggs 1 week before parturition were collected by questionnaire. Trace amounts of allergens detectable in cord blood are indicated by *. BLG, β -lactoglobulin; OVA, ovalbumin.

altered T cell function in neonates at risk of developing allergic disease [17]. Reduced IFN- γ production was found in neonates with a positive family history of atopic disease and in infants developing atopic eczema in childhood [17–19].

Allergen exposure in early childhood plays a critical role in the development of allergic responses. However, no association was found between maternal exposure to indoor allergens and allergen-induced proliferation of CBMCs and disease development at the age of 12 and 24 months [9, 10, 20]. The diaplacental transport of Fel d 1-IgG immune complexes has been suggested to provide the initial signal for T cell priming to inhalant allergens [21]. However, since immune responses to inhalant allergens are commonly detected in cord blood cells in atopic and non-atopic infants, this is not necessarily associated with the development of allergic diseases [21]. In line with this, no association was found between T cell proliferation and allergen-specific IgE levels in cord blood or between IL-5 production and T cell reactivity after house dust mite (HDM) stimulation, indicating that allergen-specific T cell reactivity in cord blood is not equivalent to an allergic immune response [20].

We measured, in addition, lymphoproliferative responses of CBMC to BLG and OVA, corresponding to the investigated placentas. The value of the stimulation index to BLG ranged from 1.6 to 19 and to OVA from 0.98 to 1.62. No correlation to the absolute antigen levels in maternal or fetal perfusate samples were found. However, while HDM- and OVA-specific IgG levels in cord blood have been shown to be positively associated with the magnitude of perinatal lymphoproliferative responses to these allergens, these relationships did not persist into later infancy. Cellular immune responses to specific allergens in late infancy are not significantly influenced by the levels of maternal allergen-specific IgG reaching cord blood [22].

Since allergic disease development often starts early in life, studying preterm and term placentas and cord blood for the presence of OVA and BLG is of particular importance to discriminate normal from disease pattern. In this study, low levels of the investigated nutritive allergens were released from placental tissue and could be detected in cord blood. Consistent with these findings, BLG and OVA were detected in human serum of healthy adults [23, 24]. Thirty minutes after oral intake of 1.2 L of milk, BLG could be detected in the sera of healthy individuals at a concentration of 0.1–3 ng/mL [23]. In our investigation, BLG and OVA were undetectable or at the limit of detection if the pregnant mother was consuming only milk and egg-containing nutriment (such as noodles, puddings, cakes), whereas after consumption of unprocessed milk and eggs both allergens could be observed in cord blood and perfusate samples. The nutritive allergens could be detected in cord blood even if the pregnant mother has not ingested milk or eggs 2–3 days before delivery. This observation may indicate that the human placenta takes up and stores allergens, which thereafter are slowly released to the fetal compartment. This assumption is consistent with the results of our previous allergen-perfusion study [11], in which large amounts up to 90% of the allergen added to the maternal circulation could neither be recovered in the fetal nor in the maternal perfusate samples, and the immunohistochemical detection of allergen amounts in the placental stroma (unpublished experiments).

The detection of OVA and BLG in preterm and term placentas and cord blood may explain how fetal T cell responses to nutritive allergens would be induced in neonates. The subsequent immunological processes *in utero* following fetal exposure to nutritive allergens are believed to be physiological. However, the possible implications for postnatal immunity with respect to the development of atopic disease or allergen/antigen-specific tolerance induction remain to be elaborated.

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