

Materno–fetal passage of nutritive and inhalant allergens across placentas of term and pre-term deliveries perfused *in vitro*

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Summary

Background The pre- and postnatal environment appears to be of crucial importance for the manifestation of allergic diseases, which often begin during infancy. Although T cell reactivity of fetal origin to a range of common allergens is present in most cord blood samples, the immunological basis remains unclear.

Objective In order to test the hypothesis of transplacental allergen transfer we studied double-sided open *ex vivo* perfusion experiments of isolated placental cotyledons with the nutritive allergens beta-lactoglobulin (BLG) and ovalbumin (OVA) and the inhalant major birch pollen allergen Bet v1.

Methods Placentas of full-term and pre-term newborns were obtained immediately after delivery to recover functionally active maternal and fetal circulations. Thus, a fetal artery and a fetal vein were cannulated and perfused with pure medium (fetoplacental circulation), whereas the intervillous space of placentas was flushed with allergen containing medium by puncture of the basal plate (maternoplacental circulation). Samples that were collected throughout the perfusion experiment from fetal venous outflow were tested by allergen-specific enzyme-linked immunosorbent assays (ELISA) for the presence of allergens indicative of materno–fetal transplacental passage.

Results We observed transplacental transfer of BLG, OVA and Bet v1 in placentas of term as well as premature deliveries. The respective allergen was readily detectable in fetal effluent at the beginning of the perfusion experiment and allergen levels reached a plateau after about 2 h. The steady state transfer rate of BLG and OVA in term placentas was $0.012\% \pm 0.001$ and $0.013\% \pm 0.001$ of total dose, i.e. 130.21 ± 7.41 ng/mL and 115.83 ± 6.07 ng/mL, respectively. The observed transfer rate of Bet v1 after 2 h of perfusion was $0.155\% \pm 0.034$ of total dose, that is 2.41 ± 1.36 ng/mL. Transplacentally transferred concentration of BLG and OVA in pre-term placentas increased continuously throughout perfusion time from 5.32 ± 0.92 ng/mL at 1 min to 87.53 ± 21.93 ng/mL at 120 min and 1.35 ± 0.31 ng/mL at 1 min to 112.87 ± 5.25 ng/mL at 150 min, respectively.

Conclusion Allergen-specific cord blood reactivity may be attributed to low levels of allergens crossing the human placenta and providing the fetus with the necessary stimulus for T cell priming.

Keywords allergy, Bet v1, BLG, IgG, OVA, placenta, transfer

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Introduction

The prevalence of allergies in the western hemisphere has increased steadily over the last decades [1, 2]. The perinatal period is assumed to be of special relevance for allergy manifestation and priming of the fetal immune system is widely thought to be associated with environmental effects, one of which was supposed to be maternal immunity [3]. As prevention of allergic sensitization is the major goal in addition to treatment of established allergic diseases, many efforts have been focused on understanding the immunological basis of materno–fetal interactions.

The presence of peripheral blood mononuclear cell (PBMC) sensitivity to allergens in neonatal blood in both atopic and non-atopic individuals has been demonstrated by several investigators [4–10]. This finding was reported from around 20 weeks of gestation [5, 11], indicating intrauterine exposure to allergens with subsequent development of memory function. However, what is the pathway of allergen exposure to the fetus?

Allergen exposure *in utero* may occur both via transfer of allergens in the amniotic fluid and via transfer across placental tissue. Our group has focused on the transplacental pathway and recently published data supporting the existence of materno–fetal allergen transfer via the placenta [12]. However, so far it is not clear (i) whether transplacental allergen transfer happens in general, (ii) at what stage (time) in pregnancy it occurs predominantly, and (iii) which transfer mechanism accounts for transplacental allergen passage. To address these questions, the objective of our study was to investigate transmembranous

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transfer of the nutritive hen's egg allergen ovalbumin (OVA) and the cow's milk whey protein beta-lactoglobulin (BLG), as well as the inhalant major birch pollen allergen Bet v1. As cord blood mononuclear cell reactivity to allergens was observed as early as 20 weeks of gestation [5, 11], the transfer experiments were performed with placentas obtained from premature and full-term deliveries. To examine the contribution of immunoglobulin G (IgG) on allergen transfer, concentrations of endogenous placental IgG and its release throughout the perfusion experiments were determined. Perfusion experiments with a short initial allergen pulse (10 min) followed by a 2-h chase should help to analyse diaplacental transport mechanisms.

Methods

Placentas

Placentas were obtained immediately after vaginal delivery ($n = 19$) or Caesarean section ($n = 25$), from normal pregnancies between 39 and 41 weeks of gestation. All mothers were reportedly healthy and did not take any medication during pregnancy other than vitamins and iron. Placentas of mothers from pre-term newborns (26, 28, 32 and 34 weeks of gestation) were obtained after Caesarean section. Studies on placentas after delivery have been approved by the institutional review board of the general hospital of Vienna.

Viability

Recently, minimal leakage was stated as best control parameter for viability of placental perfusions [13]. Thus, the integrity of placental preparation was assessed by controlling fetal flow volume with a minimum recovery of 95% (ensuring intact membranes and constant perfusion pressure in fetal vessels), as well as by constant pH of fetal elutes. In addition, viability of placental tissue was monitored by use of trypan blue, a dye which reportedly does not pass through placental membranes.

Perfusion experiments

We performed double-sided open (no recirculation of the perfusate) *ex vivo* perfusion experiments of isolated placental cotyledons as previously described [14, 15]. The preparation of the

placental tissue had to be finished within 15 to 20 min. Briefly, after an intact area on the basal plate has been selected, a branch of the umbilical artery and of the umbilical vein on the corresponding chorionic surface was cannulated. The cannulated fetal vessels were flushed with pure medium at a mean flow rate of 3 mL/min. Subsequently, the respective tissue was fixed between two plastic rings and transferred to the perfusion chamber. Following recovery of the fetal circulation five thin cannulas were inserted through the basal plate into the appropriate intervillous space supplied by these vessels. The cannulas corresponding to maternal spiral arteries were perfused at a mean flow rate of 3 mL/min with medium containing the allergens OVA (1 mg/mL), BLG (1 mg/mL) or Bet v1 (2 µg/mL). Trypan blue was added to the maternal perfusate several minutes before finishing each experiment. Perfusions were run for 2 to 3 h with 1-min samples of fetal venous outflow taken every 5 min. Samples were frozen until analysis at -20°C . Figure 1 describes the perfusion set-up.

Medium

The perfusate consisted of M199 tissue culture medium (11 g/L, Biochrom KG, Berlin, Germany) supplemented with dextran T-40 (20 g/L, Pharmacia, Uppsala, Sweden), bovine serum albumin (3 g/L, Behringwerk KG, Marburg, Germany), and sodium hydrogen carbonate 8.4% (10 mL/L, Sigma Chemical Company, St Louis, MO, USA). The medium was warmed to 37°C and pH was adjusted to 7.4.

Allergen determination by sandwich-ELISAs

Assays for detection of the allergens OVA (Grade V, Sigma), BLG (80%, Sigma), Bet v1 (Biomay, Vienna, Austria) and IgG were established or adjusted in our laboratory. All samples were tested in triplicates and a maximum intra-assay variation of 7% was accepted. At all assays p-nitrophenyl phosphate (Sigma) was used as substrate for colourimetric reactions. Absorbance was determined at 405 nm with a Spectrophotometer (Anthos 2010, Anthos Labtec Instruments, Austria). Human IgG (Octagam®, Octapharma, Vienna, Austria) and the allergens BLG, OVA and Bet v1 diluted in medium served as positive control. Concentrations of allergens and immunoglobulins were determined by extrapolation from the respective standard curve.

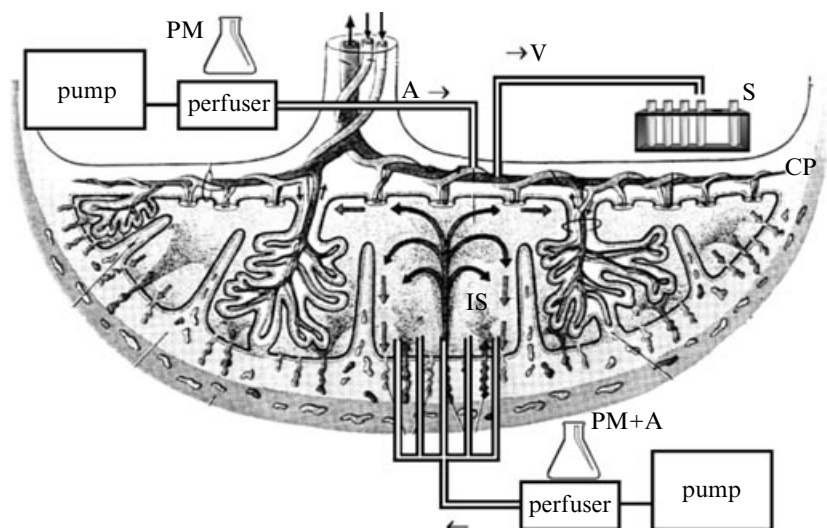


Fig. 1. Schematic diagram of the *ex vivo* dual perfusion system. Fetal circulation: After cannulation of an artery (A) and the corresponding vein (V) at the junction to the chorionic plate (CP), fetal vessels were rinsed with perfusion medium (PM). Maternal circulation: Five cannulas were placed in the intervillous space (IS) and were flushed with perfusion medium supplemented with the respective allergen (PM + A). Samples (S) of fetal venous outflow were collected in regular time intervals throughout the perfusion experiment. Arrows indicate flow direction.

Beta-lactoglobulin ELISA Plates were coated with polyclonal rabbit-anti-BLG. Blocking with human serum albumin HSA (Sigma) was followed by an overnight incubation with samples and standards. Immune complexes were detected with rabbit-anti-BLG conjugated with alkaline phosphatase (both Ab from Bethyl Laboratory Inc., Montgomery, TX, USA). A concentration linearity for BLG standard was found between 0.2 and 100 ng/mL.

Ovalbumin ELISA After coating of microplates with monoclonal goat-anti-OVA, subsequent blocking with bovine serum albumin BSA (Sigma) and overnight incubation with samples the allergen-antibody complex was captured by rabbit-anti-OVA (both Ab are Cappel Ab from ICN Pharmaceuticals Inc., Costa Mesa, CA, USA). Swine-anti-rabbit labelled with alkaline phosphatase (Sigma) was used as detecting Ab. The sensitivity of the assay was 25 pg/mL.

rBet v1 ELISA The following antibodies were used: mouse-anti-rBet v1 as coating Ab, rabbit-anti-rBet v1 as second Ab (both kindly supplied from Department of Pathophysiology, Dr Bohle, University of Vienna, Austria) and swine-anti-rabbit conjugated with alkaline phosphatase (Sigma) for the detection of the immune complexes. A concentration linearity for Bet v1 standard was found between 0.5 and 25 ng/mL.

ELISA for IgG Coating of plates was performed with Fc-specific mouse-anti-hIgG. After an overnight blocking step with gelatine (Merck kgaA, Darmstadt, Germany) immune complexes were detected with F_{ab}-specific goat-anti-hIgG

labelled with alkaline phosphatase (both Ab provided from Sigma). The assay has a sensitivity of 0.5 ng/mL.

ELISA for IgE Assays for total IgE (KallestadTM total IgE Microplate, Bio-Rad Laboratory, Munich, Germany) were performed in accordance with the manufacturer's instructions. Immunoglobulin E is detectable from 0.35 IU/mL upwards.

Results

In summary, 48 preparations used for perfusion experiments fulfilled our perfusate recovery criteria and were included in the study.

Transplacental allergen transfer

Ovalbumin, beta-lactoglobulin and Bet v1 occurrence in fetal effluents could be detected in all perfused placentas of term (Fig. 2) and pre-term deliveries (Fig. 3).

Term deliveries Allergens are readily detectable in fetal outflow at the beginning of the perfusion experiment. The fetal concentration of BLG increased from 3.07 ± 1.02 ng/mL at 1 min to 126.26 ± 6.54 ng/mL at 2 h. At perfusions with OVA a comparable increase in allergen transfer with 2.42 ± 1.86 ng/mL at 1 min to 115.22 ± 7.11 ng/mL at 140 min was observed. Thereafter, a steady state level was reached. Detectable concentrations of Bet v1 in fetal circulation increased from 0.103 ± 0.097 ng/mL at 1 min to 2.41 ± 1.36 ng/mL.

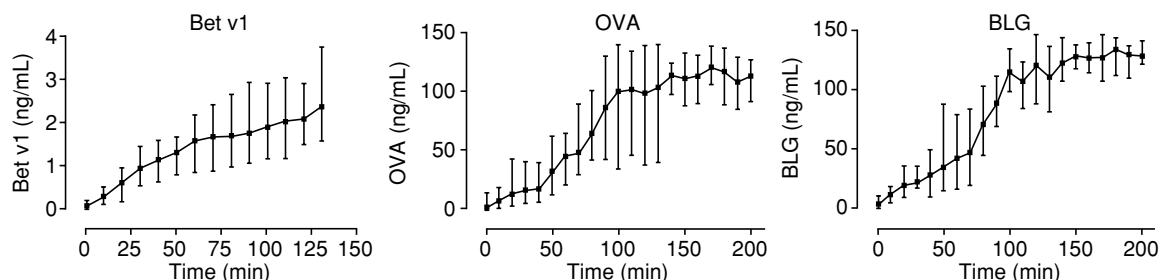


Fig. 2. Unidirectional *ex vivo* materno-fetal transfer of beta-lactoglobulin (BLG), ovalbumin (OVA) and Bet v1 in placentas of full-term newborns. Maternal perfusion medium was supplemented with the nutritive allergens at a concentration of 1 mg/mL and the inhalative allergen at a concentration of 2 µg/mL. Allergen concentrations appearing in fetal circulation were determined by allergen specific ELISAs ($n_{\text{BLG}} = 10$; $n_{\text{OVA}} = 11$; $n_{\text{Bet v1}} = 11$). Values are means $\pm$ SEM.

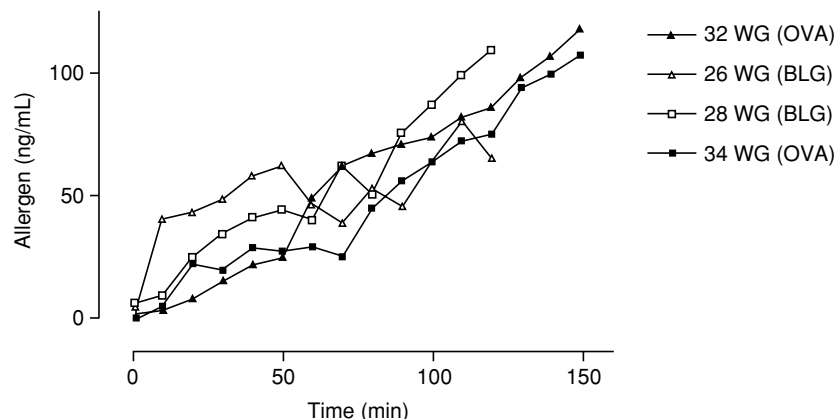


Fig. 3. Unidirectional *ex vivo* materno-fetal transfer of beta-lactoglobulin and ovalbumin in placentas of pre-term newborns. The intervillous space was flushed with medium containing the allergen at a concentration of 1 mg/mL. Detection of the respective allergen in samples of fetal outflow was performed by allergen specific ELISAs ($n_{\text{BLG}} = 2$; $n_{\text{OVA}} = 2$). Values are means $\pm$ SEM.

Steady state unidirectional materno-fetal ratio of BLG and OVA transfer ($F:M$) * 100 (F = mean allergen concentration measured in fetal outflow, M = allergen concentrations applied to maternal perfusate) was 0.012 ± 0.001 and 0.013 ± 0.001 , respectively. The transfer ratio for the inhalant allergen Bet v1 calculated after 2 h of perfusion was 0.155 ± 0.034 .

Premature deliveries From 26 weeks of gestation upwards we could demonstrate placental passage of both nutritive allergens. Fetal concentrations of BLG and OVA in pre-term placentas increased from 5.32 ± 0.92 ng/mL to 87.53 ± 21.93 ng/mL and 1.35 ± 0.31 ng/mL to 112.87 ± 5.25 ng/mL, respectively, after 2 h of perfusion.

Immunoglobulin G (IgG) release into the fetal effluent Analysis of perfusion samples showed high release of endogenous maternal IgG into the fetal circuit, with initial concentrations of 16760 ± 5980 ng/mL decreasing to 250 ± 140 ng/mL at the end of the perfusion experiment at 3 h (Fig. 4).

Pulse-chase experiments At the beginning of the chase period following an allergen pulse of 10 min we observed further

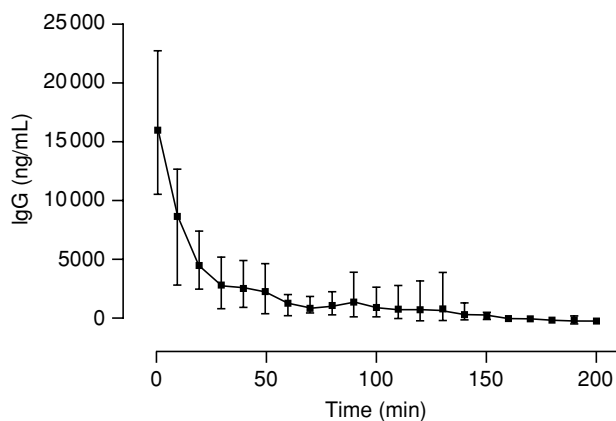


Fig. 4. Release of endogenous immunoglobulin G (IgG) into fetal circulation. Washout levels of endogenous IgG throughout perfusion experiments in placentas of full-term deliveries were determined by IgG-specific ELISA ($n=4$). Values are mean $\pm$ SEM.

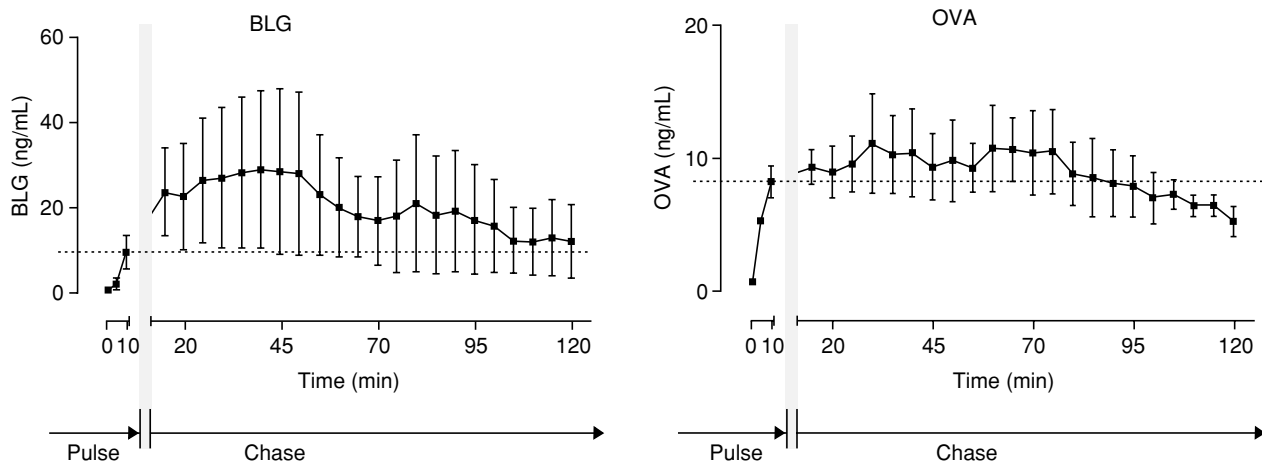


Fig. 5. Perfusion experiments with an allergen pulse followed by a 2-h chase period. Perfusion experiments were performed in placentas of full-term newborns. Allergen concentration added to maternal perfusate at the initial allergen pulse of 10 min was 1 mg/mL. Throughout the chase period the intervillous space was flushed with medium containing no allergen. Shown values are detected by ELISA and are means $\pm$ SEM ($n_{BLG}=2$; $n_{OVA}=2$).

increase in BLG concentration in fetal circulation. At the end of the 2-h chase period, BLG levels in fetal outflow decreased moderately and came up to about the level observed at the end of the pulse period. Similarly, data generated with OVA showed an increase of fetal allergen levels at the beginning of the chase period; however, it was more slightly than seen in BLG perfusion experiments (Fig. 5).

Discussion

A major issue in the question of early allergy prevention is whether and how allergens encounter the fetal immune system. Several studies evaluating antigen-specific proliferative responses of cord blood cells [4–10] indicate that the fetus is exposed to allergens or allergenic fragments. One of the ways prenatal allergen contact might occur is via the placenta. We focused exclusively on transplacental passage by performing *ex vivo* perfusion experiments and could detect the nutritive allergens OVA and BLG in fetal outflow of all studied placentas. Contrary to a previous study [12], we could demonstrate universal transplacental transfer of the inhalant allergen Bet v1 by improving the Bet v1-ELISA system (Fig. 2). In accordance with data supporting allergen-specific T cell reactivity from 20 weeks of gestation [5, 11], we observed diaplacental allergen transfer of OVA and BLG in placentas of premature deliveries (Fig. 3). Use of the perfusion system with pre-term placentas was limited by the small size of placental vessels. Thus, we could show transplacental allergen transfer as early as 26 weeks of gestation, although transfer might occur even earlier. In four additional experiments the maternal perfusate was supplemented with pooled sera from atopic individuals with a total IgE level of 189 IU/L. We observed no materno-fetal transfer of IgE at any time of the perfusion, thus attesting functional integrity of the materno-fetal interphase (data not shown). Although supplied allergen concentrations are relatively high and experiments performed with isolated placental tissue create an artificial situation, we observed transfer concentrations of the inhalant allergen Bet v1 comparable with recently published levels of house dust mite allergen measured in cord blood [16].

Similar transplacental transfer rates have been demonstrated in rats after feeding of dietary antigens [17].

At pulse-chase experiments we observed increasing levels of BLG and OVA in fetal circulation well after discontinuation of the pulse period (Fig. 5). This finding may be attributable to the high allergen doses the intervillous space was loaded with during the allergen pulse. Binding of proteins within the placenta itself was recently proposed, thus placental protein binding may retard or prevent the transfer [18]. Differences in transfer kinetics between BLG and OVA observed at pulse-chase experiments may be due to the molecular weight.

The molecular weight, lipid-solubility, polarity and degree of ionization at physiological pH are significant factors influencing transplacental passage. Glyburide, for example, a small (494 D), un-ionized and lipid-soluble molecule is thought to cross the placenta only sparsely due to very extensive plasma protein binding [18]. Regarding our experiments, variations in materno-fetal transplacental allergen transfer ratios calculated for nutritive and inhalant allergens may be explained by varying molecular weights (OVA = 43 kDa, BLG as dimer = 38 kDa, Bet v1 = 17 kDa) and/or different pI of allergens (OVA = 4.7, BLG_A = 5.14, BLG_B = 5.31, different isoforms of Bet v1 in the range of = 4.9 to 5.9). Poor transfer of the macromolecule bovine serum albumin (68 kDa) and the glycoprotein thyrotropin-stimulating hormone (28 kDa) was recently supposed to occur by simple diffusion [19, 20]. Transfer kinetics shown for bovine serum albumin were comparable with our results observed with nutritive allergens.

Physiological data indicate that both a transcellular and a paracellular pathway are available for transfer across the human placenta [21, 22]. The two cell layers forming the exchange barrier are the fetal endothelium and the syncytiotrophoblast. Contrary to the endothelium [23], the syncytium has no lateral cell membranes forming an intercellular space. However, the continuous structure of the syncytiotrophoblast is interrupted by degeneration. The gap is immediately filled by fibrinoid, a regular finding in every placenta. These foci of fibrinoid account for $\geq 7\%$ of the villous surface of the normal human term placenta [24]. Recently, permeability for macromolecules through the syncytiotrophoblast discontinuities on placental villi has been reported for horseradish peroxidase (40 kDa) [25] and alfafetoprotein (40 kDa) [26].

Alternatively, the syncytium can be passed transcellularly. It is well known that IgG crosses the placenta via the neonatal Fc receptor FcRn expressed on syncytiotrophoblast and endothelial cells [27–29] from early in gestation, becoming maximal in the last weeks of pregnancy [30]. In accordance with these data we could demonstrate a strong release of maternal endogenous IgG into the fetal circulation (Fig. 4). As maternal IgG levels are related to exposure [31] one might expect high levels of IgG to the nutritive allergens BLG and OVA in sera from mothers. In addition, it was demonstrated that virtually all cord blood samples are rich in maternally derived IgG subclass Abs against common environmental allergens [32, 33]. Thus, transplacental transfer of antibody-antigen complexes from the mother to the fetus may be plausible and has been repeatedly suggested [19, 27, 34]. This theory is substantiated by the detection of immune complexes in cord blood [35].

The results of our *in vitro* perfusion model indicate that every unborn baby may be exposed to tiny amounts of the ubiquitous nutritive allergens OVA and BLG and occasionally to the

seasonal birch pollen allergen Bet v1 at least from the 26th week of gestation. Despite high molecular weights of the studied allergens we speculate that transplacental allergen transfer mainly occurs by a paracellular route; however, transfer of IC is conceivable as well. So far definitive proof is lacking but the necessary investigations are underway.

Our observation implies that fetal allergen exposure is a common phenomenon, which challenges the conventional opinion that the unborn should be protected from allergens. Therefore we suppose the presence of allergens *per se* in fetal circulation may in fact be part of the normal development of immunity. As pregnancy is a Th2 phenomenon [36, 37] and allergy is characterized by a Th2 dominance, the postnatal development of a balanced Th1/Th2 immunity is essential. A critical step may be the acquisition of the appropriate commensal gut flora after birth, which is characterized by a skew towards Th1 [38–40]. Thus, other factors enhancing immune deviation after birth when the newborn faces numerous allergens again seem to be of crucial importance for the atopic outcome.

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