

ORIGINAL PAPER

Most of diaplacentally transferred allergen is retained in the placenta

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Clinical and
Experimental
Allergy

Summary

Background Transplacental transfer of nutritive and inhalant allergens has been described being potentially responsible for a series of events leading to antigen-specific immune responses in the fetus. As such, cord blood T cell responses appear ubiquitously. However, studies failed to reveal a consistent dose–response relationship between antenatal allergen exposure and allergen-specific cellular reactivity in cord blood.

Objective To examine the transfer process of allergens (ovalbumin (OVA), β -lactoglobulin (BLG), birch pollen allergen Bet v1) in placental tissue (BeWo cell line, *ex vivo* placenta model).

Methods The choriocarcinoma cell line BeWo was used to study the allergen uptake and transfer experiments *in vitro*. In the *ex vivo* placenta model the contribution of different placental compartments was evaluated. For this, immuno-histochemistry, immuno-electronmicroscopy and ELISA techniques were applied using monoclonal antibodies to Bet v1, OVA and -BLG.

Results *In vitro* transfer studies on a BeWo cell-layer revealed an intracellular allergen uptake and a trans-trophoblastic allergen transfer, which was temperature- and concentration dependent, pH sensitive and asymmetric. Allergen-specific staining of placental tissue after allergen perfusion (BLG) demonstrated bulk of the allergen in the syncytio-trophoblastic cell layer and minor staining in the villous stroma and in the endothelium of fetal vessels. Immunogold staining revealed an accumulation of the perfused allergen in the trophoblastic basement membrane.

Conclusion *In vitro/ex vivo* trans-trophoblastic and trans-placental allergen transfer is shown with an accumulation of most of the allergen in placental tissues, potentially explaining the missing direct dose–response relationship between prenatal (maternal) allergen exposure and allergen-specific cellular reactivity in cord blood.

Keywords allergen transfer, allergy, antibodies, human, placenta

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Introduction

The prevalence of atopic diseases steadily increases and the disease manifestation often starts early in infancy [1–6]. Thus, the immunity of the mother during the perinatal period is thought to play a decisive role in the atopy development of the newborn [5, 7–9]. This notion is in line with the observation that the atopic status of the mother has a stronger influence on the atopic outcome of the baby compared with the atopic history of the father [10, 11]. Moreover, allergen-specific reactivity of cord blood derived mononuclear cells [12–16] has been shown ubiquitously as a basis for an immunological interaction of the fetal immune system with exogenous allergens.

However, studies failed to reveal a dose–response relationship between antenatal allergen exposure and cord blood cell reactivity.

The materno-fetal transmission of immune mediators such as allergens, immunoglobulins or cytokines [17, 18] have been discussed as eliciting factors of type I allergy. Recently, transplacental transfer of nutritive and inhalant allergens from the maternal to the fetal blood circulation in placentas of term and pre-term deliveries was demonstrated by the help of an *ex vivo* open placenta perfusion model [19–23].

The human placenta is the primary site of nutrient and gas exchange between the mother and the fetus. Nutrients and other substances ingested by the pregnant mother,

which appear in the fetal circulation, have to pass through several biological placental barriers. The first selective membrane is the syncytiotrophoblast. This epithelial-like multi-nucleated layer without separating cell borders extends over the surface of all villous trees as well as over parts of the inner surface of chorionic and basal plate. Furthermore, the villous stroma and the endothelium of fetal capillaries within the villous trees must be passed to get into the fetal blood stream [24]. As it is well known that immunoglobulin G (IgG) provides the baby with passive immunity during pregnancy and the first weeks of life, the interaction of allergens with maternal allergen-specific IgG as antigen-antibody complexes may interfere with the free passage of allergens. Allergen-IgG immune complexes have been detected in cord blood and sera from allergic and non-allergic mothers [25], thus a transplacental transfer of allergens via the neonatal IgG receptor (FcRn) expressed on syncytiotrophoblast and on fetal vessel endothelium in placental tissues [26, 27] has been suggested. However, the relevance of IgG on diaplacental allergen transfer is still speculative.

We recently demonstrated in an *ex vivo* model that transplacental transfer of nutritive and inhalant allergens occur. Interestingly, most of the allergen applied did not cross the feto-maternal interface and the place of deposition remained undetermined. This raised the question of delineating the track of the allergen from the mother to the fetus defining compartments limiting the materno-fetal allergen transfer. By the use of BeWo cell lines and the *ex vivo* placenta perfusion model great majority of allergen could be assigned to the syncytiotrophoblast cell layer with the basement membrane as major allergen-trapping region.

Materials and Methods

Allergens

β -Lactoglobulin (BLG) and ovalbumin (OVA) have been purchased by Sigma Chemicals (St Louis, MO, USA) of 95% purity. Bet v1 was purchased by Biomay (Vienna, Austria) at > 98% purity. Lipopolysaccharide (LPS) contamination was measured by the kinetic turbidimetric limulus assay and LPS contamination was cleared by Pierce column application. Allergen concentrations were used according to published data [19, 20] based on the detection limits of allergen-specific ELISAs.

Cell culture

The choriocarcinoma BeWo cell line (clone b24 kindly supplied by I. Ellinger and R. Fuchs, Department of Pathophysiology, Medical University of Vienna, Austria) was cultured in 75 cm² flasks (Costar, Cambridge, MA, USA) at 37 °C under 5% CO₂ and 95% humidity at pH 7.4 as described [28]. Cells were passed either on 12-well

culture plates (BD, San Jose, CA, USA) for intracellular uptake studies or on transwell filter inserts with PET membranes (BD) for transfer experiments. The immortalized cytotrophoblast cell line BeWo has been repeatedly used in the literature because trophoblast cells represent the very first cellular barrier in placental tissue. In addition, the transwell system displays an ideal model to study the translocation of substances across polarized cells grown on permeable filter inserts [29]. Moreover, BeWo cells serve as a valid and convenient system to study the cellular activities and transplacental transport processes.

For uptake experiments BeWo cells were grown on 12-well culture plates pre-incubated with L15⁺ (Leibovitz 15 medium supplemented with 1000 mg/L glucose, 10 mM HEPES and 0.5% bovine serum albumin) for 30 min at 37 °C. After washing, cells were loaded with various concentrations of allergens at 37 °C or on ice for different time periods. After several washing steps, cells were lysed with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at 4 °C and the amount of allergen in cell-free supernatant was quantified with allergen-specific ELISAs.

For transport experiments, BeWo cells grown confluent on transwell filter inserts (0.45 μ m pore size, high pore density, 0.8 cm², BD) were pre-incubated with L15⁺ for 30 min at 37 °C. After rinsing with PBS⁺, various concentrations of allergen were added to the apical or basolateral chamber side at 37 °C or on ice. Samples from the opposite side of the chambers were taken at scheduled time-points and analysed with allergen-specific ELISAs. Data represent four independent experiments, each performed in triplicates. For intracellular uptake and transfer studies the allergens OVA, BLG and Bet v1 were used.

To confirm tightness and confluence of BeWo cells on inserts trans-epithelial electrical resistance (TEER) was measured by the Millicell[®]-ERS system (Millipore Corp., Bedford, MA, USA). Resistance expressed in Ω /cm² was calculated by subtracting blank resistance from sample resistance with correction for the area covered by the cell layer $[(R_{\text{sample}} - R_{\text{blank}}) \times \text{area}_{\text{filter}} \text{ cm}^2]$. Furthermore, ³H-Mannitol was applied apically as a paracellular transfer marker. A permeability of the hydrophilic substance of less than 2% after 1 h of incubation at 37 °C determined by a β -scintillation counter was regarded as a good indicator of a tight cell layer.

To follow cell growth and formation of confluent cell layers with BeWo-cells, initially several transwell filter inserts were cut from the 5th to 10th day post-seeding. The cells on the membrane were formalin fixed, paraffin embedded and subsequently stained with haematoxylin and eosin according to standard procedures.

Enzyme linked immunosorbent assays

Sandwich-ELISAs for detection of the allergens OVA, BLG, and Bet v1 have been established and used as previously

described [30]. In brief, for OVA detection coating antibody goat anti-chicken OVA (ICN, Cappel, CA, USA) and capturing antibody rabbit anti-OVA (ICN) with alkaline phosphatase swine anti-rabbit Ab (DAKO, Glostrup, Denmark) were used. For BLG detection rabbit anti-BLG Ab (Bethyl, Montgomery, TX, USA), alkaline phosphatase conjugated rabbit anti-BLG (Bethyl) was applied (detection limit: 200 pg/mL). For the Bet v1 Sandwich-ELISA mouse anti-Bet v1 and rabbit anti-Bet v1 (both Abs kindly supplied by B. Bohle, Department of Pathophysiology, Medical University of Vienna, Austria), swine anti-rabbit Ab labelled with alkaline phosphatase (DAKO). For human IgG detection mouse anti-human IgG F_c -specific Ab (Sigma Chem. Co.) and goat anti-human IgG F_{ab} -specific Ab labelled with alkaline phosphatase (Sigma Chem. Co.) was used (detection limit: 0.5–125 ng/mL). The substrate *p*-nitrophenyl phosphate was used for colorimetric reactions in each ELISA test system. The absorbance was determined at 405 nm.

Placenta perfusion technique

Placentas from uncomplicated pregnancies were obtained after elective caesarean section at term (> 37 weeks of gestation). The fetal and the maternal circulations of the whole placenta were reconstituted using an open, *ex vivo*, perfusion model as previously described [19, 31]. In brief, an artery and the corresponding vein on the chorionic plate were cannulated, connected to a pump and immediately flushed with aerated medium pre-warmed to 37 °C (fetal circulation). Thereafter, five thin catheters were passed through the basal plate to supply the intervillous space with medium (maternal circulation). Viability of placental tissue was monitored as described previously [21] with additional assessment of lactate production and glucose consumption during the experimental phase and measurement of maternal to fetal transmission of the freely diffusable substance antipyrin as described [32]. Studies on placentas after delivery have been approved by the institutional review board of the Medical University of Vienna.

Immuno-histochemistry - allergen localization studies

Placentae of term deliveries were perfused for 1 h with different concentrations of the allergen BLG (20, 2, 0.2 and 0.02 mg/mL). After finishing perfusion the whole placenta was fixed in 4.5% buffered formalin. Histological sections were deparaffinized in xylol. Antigen retrieval was performed by incubation in 0.1% protease solution (Protease XIV, Sigma Chem. Co.) for 10 min followed by incubation in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. Slides were incubated for 30 min with polyclonal affinity purified rabbit antibodies against BLG (Bethyl) in a dilution of

1 : 80. Visualization of bound antibodies was performed using Dako ChemMate TM Detection Kit peroxidase/DAB rabbit/mouse according to the manufacturers instructions. 3-amino-9-ethylcarbazole (BioGenex, San Remo, CA, USA) was used as chromogene.

Immuno-electronmicroscopy

Immunogold electronmicroscopy was performed on ultrathin sections of BLG-perfused as well as non-perfused placenta tissues following a protocol as described previously [33]. In brief, pieces of tissue were fixed in 0.1% distilled glutaraldehyde (Merck, Darmstadt, Germany) in 100 mM phosphate buffer, pH 7.2, and were embedded in Lowicryl K4M resin. A polyclonal affinity purified anti-BLG-antibody (Bethyl) was used as primary antibody in a dilution of 1 : 80. Ultrathin sections were labelled by an indirect immunogold protocol. In control experiments, incubation with primary antibodies was omitted.

Statistical analysis

Results are presented as the mean. Data were compared using the Mann-Whitney *U*-test (unpaired groups) or the Wilcoxon signed rank test (paired groups). A value of $P \leq 0.05$ was considered statistically significant.

Results

Trans-trophoblastic allergen transfer is an active process

BeWo cells were incubated with allergens at various concentrations at 37 °C or on ice at pH 6.0 or 7.4 to investigate if allergens are transported in a transcellular or a paracellular way. At scheduled time-points the intracellular compartment and the supernatants were analysed for the respective allergen by ELISA. In case of allergen exposure (OVA, BLG or Bet v1) we observed an intracellular allergen uptake for all three allergens, which was concentration- and temperature dependent as defined via a significant reduction of the allergen uptake at 4 °C compared with 37 °C (Fig. 1a). Intracellular uptake of the allergens was higher at pH 7.4 than at the supra-physiological pH 6.0 (Fig. 1b) indicating that allergen uptake is mediated rather by fluid phase mechanism than by receptor processes.

To define the polarity of allergen trafficking an *in vitro* transwell system was used. Tightness of the monolayer was assured via measurement of the TEER at 6–7 days post-seeding and a value of $55.4 \pm 7.86 \Omega/\text{cm}^2$ was representative for a confluent cell monolayer. Incubation of BeWo cells over a longer time period resulted in formation of multiple cell layers as detected by cross-sections (data not shown). Similar to intracellular uptake studies we observed a trans-trophoblastic allergen transfer, which

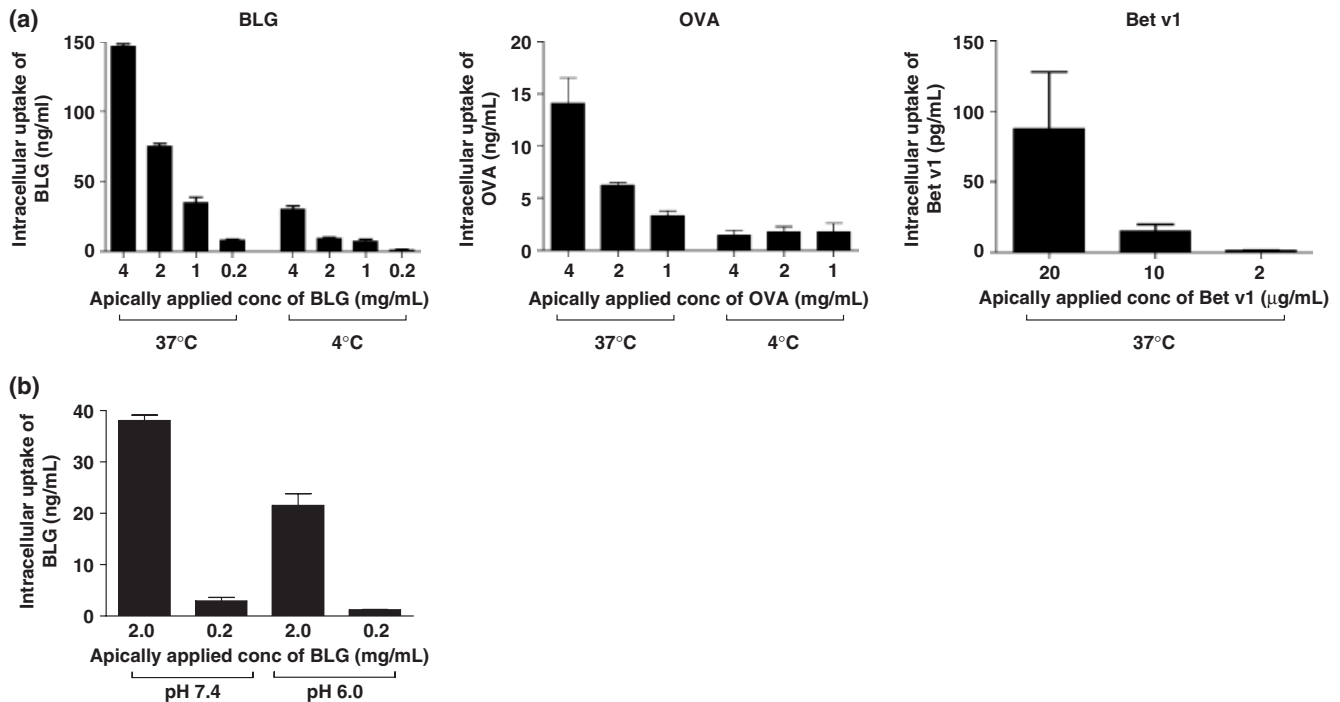


Fig. 1. Monolayers of the trophoblast cell line BeWo grown in 12 well plates were exposed to allergen at various concentrations, temperature and pH. Intracellular uptake of ovalbumin (OVA), Dose-response-lactoglobulin (BLG) and major birch pollen allergen Bet v1 by BeWo cells is temperature- and concentration dependent (a). In addition, internalization is pH-sensitive as demonstrated for the major milk allergen dose-response-lactoglobulin (BLG) (b). The intracellular allergen uptake was measured after a 10 min incubation period. Following lysis, supernatants were analysed by allergen-specific ELISA.

was concentration- and temperature dependent showing a significantly reduced transfer at 4 °C compared with 37 °C (Fig. 2). Polarization of the cells affected the allergen transfer. Allergen transfer was significantly more pro-

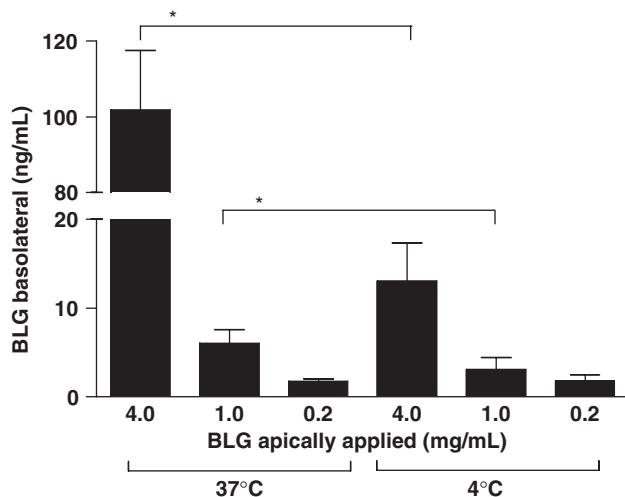


Fig. 2. Trans-trophoblastic allergen transfer of dose-response-lactoglobulin (BLG) across polarized BeWo cells in dependence of concentration and temperature. The apical to lateral transport was measured after 10 min of incubation with the allergen, followed by analysis of allergen content in the lateral chamber by allergen-specific ELISA.

nounced with allergen applied to the basolateral side of the filter inserts than vice versa (not shown). This observation accounts for the fact that the syncytium is a gate keeper for proteins, lipids, vitamins and gases being transferred from the mother to the fetus as well as vice versa. Some products, such as steroid hormones or α -feto protein are preferentially secreted by the fetus and thus appear at higher concentration in the maternal than the fetal circulation.

Immuno-histochemistry and immuno-electronmicroscopy trace the allergen transfer process

To track the fate of the allergen *in vivo*, staining of allergen-perfused placental tissues was performed. A marked allergen-specific staining of the first selective placental barrier, the syncytiotrophoblast was observed (Fig. 3) characterized by an accumulation of the nutritive allergen BLG in the syncytiotrophoblast layer covering the villous trees. This was evident in all perfusion experiments from the highest (20 mg/mL, Fig. 3a) to the lowest allergen concentration (20 µg/mL, Fig. 3c). The allergen-specific signal in the marginal zones of the villous tree was very intense. However, also weak BLG-specific staining was detectable in villous stroma and in endothelium of fetal vessels indicating that the allergen is apparently crossing the syncytiotrophoblastic barrier (Fig. 3a, arrow).

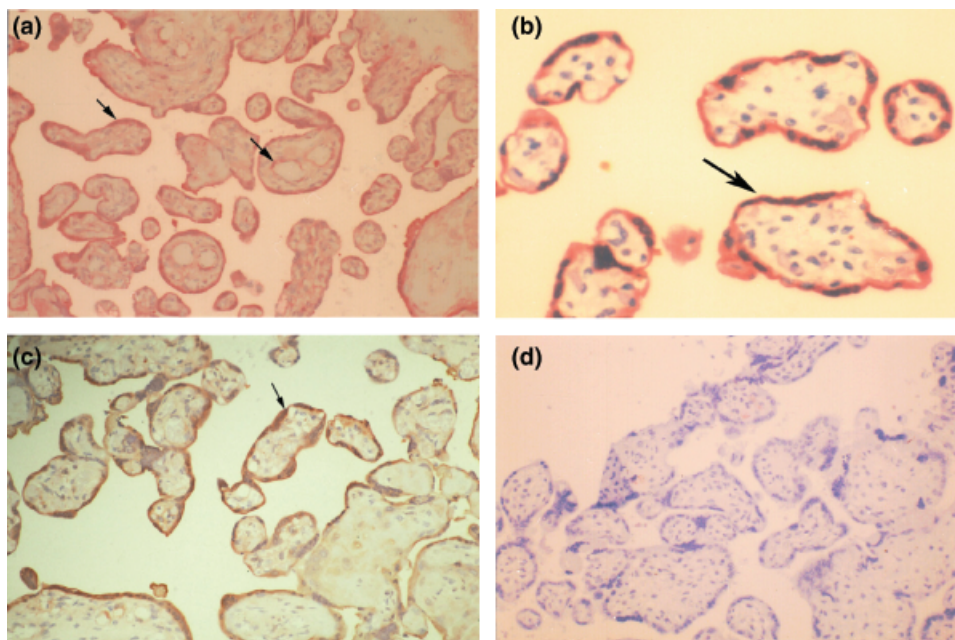


Fig. 3. Immuno-histochemical localization of BLG in sections of chorionic villi following placental perfusion with 20 mg/mL (a), 2 mg/mL (b) and 0, 2 mg/mL (c) of the allergen BLG which was added to the perfusion medium and supplied via the maternal circulation. Immuno-histochemical processed placental tissue without allergen perfusion and haematoxylin staining (d) served as control. Arrows indicate allergen-specific staining. $n_{\text{placenta}} = 2$ for each concentration. Pictures a, c and d were taken at $\times 20$, picture b at $\times 40$.

To substantiate the results from immuno-histochemistry and to determine the fate of allergens in placental tissue at a molecular level we performed immuno-electron-microscopy staining. Comparable to the results of immuno-histochemistry allergen-specific staining revealed BLG adhering to the villous syncytial membrane. In some cases allergen was detected in multi-vesicular bodies although it was also spread diffusely in villous stroma. Interestingly, bulk of the perfused allergen accumulated in the trophoblastic basement membrane (Fig. 4), indicating that this cell compartment is definitively essential for the decision of when and how the allergenic molecule will be delivered to the fetal compartment.

Discussion

This study strengthens the occurrence of a diaplacental allergen transfer and more specifically delineates for the first time that most of the allergen is retained in selective compartments in the placenta rather than directly appearing in the fetal blood circulation. The allergen transfer is an energy-dependent process. The allergen transfer data generated in this study are in line with the hypothesis that maternal exposure to a certain allergenic environment induces an allergen-specific immune response in the fetus. This has been extensively shown by many groups, for many allergens and under different culture conditions [12–14, 16, 34, 35]. The origin of this immune response has been assigned to the fetal compartment [15] suggesting that the allergen must reach the fetal compartment. In

other experimental settings allergen could be detected in cord blood and amniotic fluid. Thus, Uthoff et al. [9] applied OVA to pregnant Balb/c mice and detected immunoprecipitated OVA in cord blood serum and amniotic fluid. Similarly, Holloway et al. [36] detected Der p1 in fetal cord blood and amniotic fluid. Still unsolved is the fact that the level of allergen the mother is exposed to during pregnancy does not correlate with the fetal allergen-specific immune response [37, 38]. As such, it has been shown, that despite a strong maternal exposure to grass or birch pollen allergens during the pollen season, the fetal immune response to these allergens is not detectable. In the same way fetal immune responses to allergens could be measured without maternal exposure to these allergens. This missing dose-response relationship prompted us to investigate the mechanism of allergen transfer in more detail. For this, we used the cytotrophoblast cell line BeWo because trophoblast cells represent the very first cellular barrier in placental tissue. In this system we demonstrated that allergen transfer occurs, and is driven by an energy-dependent active mechanism. The influence of reduction of energy (4°C) and change in homeostasis (pH) clearly support this statement. Trophoblast cells represent just one selective placental barrier, excluding the stroma and the fetal endothelium. We thus aimed to confirm our cell culture observations in the more complex system of the human placenta. Following allergen perfusion with BLG by the maternal placental circulation in a placenta perfusion model [19–22], which has been recently established in our laboratory, sections were

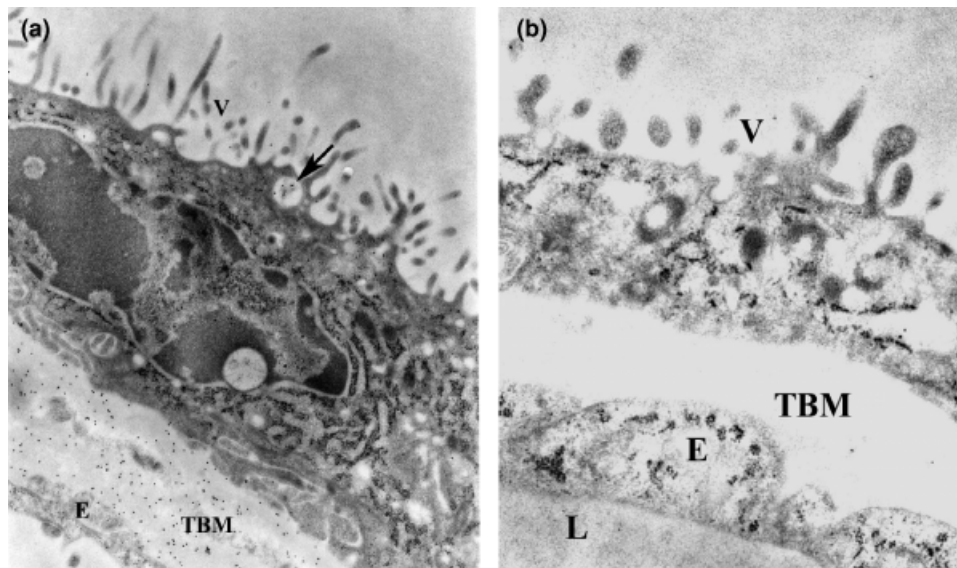


Fig. 4. Trafficking of BLG in placental tissue after BLG perfusion assessed by immunogold electronmicroscopy. BLG (2 mg/mL) was added to the medium and supplied via the maternal circulation (a). Placental tissue without prior allergen perfusion served as control (b). Arrow = multi-vesicular body, TBM = trophoblastic basement membrane, E = endothelium of fetal vessels, V = villi, L = lumen of fetal vessels; $n_{\text{placenta}} = 2$. Magnification $\times 5000$.

submitted to anti-BLG staining. As allergen transfer has been readily shown previously [19–22], immuno-histochemistry staining was only performed with the model allergen. Here, we observed allergen-specific staining in endothelium of fetal vessels and in cellular villous matrix although the greater part of the allergen was bound to the surface of the villous trees. The high amount of allergen accumulating in the syncytiotrophoblast layer independently of the allergen concentration in the perfusion fluid strongly suggests that transtrophoblastic transfer is primarily limited through syncytiotrophoblast cells. Thus, we hypothesize that only tiny amounts of allergen gradually cross the materno-fetal barrier and reach the fetal circulation. This hypothesis is supported by pulse-chase experiments performed in the same model showing continuous low-level allergen release into the fetal circulation well after stopping of the allergen supply [21]. Finally, using the *ex vivo* placenta model in previous studies we observed that despite the fact of readily detectable allergen transfer most of the allergen is not transferred to the fetus [19,21,22]. In nominal terms only around 1/2000 to 1/10 000 of the maternally applied allergen molecules would be detected in the fetal circulation. This observation, in turn, could explain why many studies tempting to elucidate a dose–response relationship between maternal allergen exposure and fetal T cell immune response development failed to show any evidence for this assumption. If allergen delivery by the syncytiotrophoblast cell layer is of such restrictive format, researchers should not expect to find a direct dose–response effect [12, 37–41].

As shown by electronmicroscopy the majority of the allergen accumulated within the trophoblastic basement membrane. However, allergen-specific signals have also

been observed at the microvillous membrane of the syncytiotrophoblast. On the basis of these investigations, the basement membrane appeared to represent a further limiting structure for allergen passage through the villous part of the placenta. Corresponding results have been published for the materno-fetal barrier of the canine placenta with a particularly high density of IgG in the basement membrane [42]. The trophoblast basal lamina with its main components collagen IV, laminin, and heparan sulfate as the glycosaminoglycan moiety of proteoglycan [43–45] has already been supposed to be a filter barrier between the maternal and fetal blood circulations comparable to the molecular sieve function of the glomerular basal lamina [24].

Our data would support the idea that in addition to humoral and cellular maternal components [17], placental and fetal factors including activation signals and apoptosis [46], release of soluble factors (not defined in our study) may regulate the fetal allergen-specific immune response. In a very recent study, Thornton et al. [46] outline the possible role of ‘thymic emigrants’ as being responsible for the ubiquitous non-memory T cell reactivity to antigens/allergens in cord blood, an alternative sound explanation for a missing dose–response relationship.

With the results of our study we conclude that an active mechanism is operative in trophoblastic intracellular allergen uptake and subsequent transfer across the complex structure of placental tissue. Bulk allergen will then adhere to the microvillous membrane of the villous tree and the trophoblastic basement membrane. Thus, we could argue, that allergen reaches the fetus, though in very small amounts, and that most of the allergen is

retained in cytotrophoblast cells. The small amount of allergen would represent the basis of the well-established allergen-specific T cell response in cord blood. Considering the expected allergen levels in the environment and thereafter in the blood circulation (to be assumed in the range of pg to ng), most allergenic molecules will be blocked in the placental tissue. The fate of the very small amount of allergen reaching the fetus is unknown but may include tolerance induction or sustained immunity.

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