

Detection of Fel d 1–immunoglobulin G immune complexes in cord blood and sera from allergic and non-allergic mothers

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It is an established fact that T-cell responses of fetal origin to inhalant allergens are present in most cord blood samples. These immune responses could be explained by trans-placental passage of peptides, either as free antigens or in complexes with immunoglobulin G (IgG), providing the fetus with a trigger for priming the T-cell system already present *in utero*. The aim of this study was to investigate the presence of the major cat allergen, Fel d 1, in complexes with IgG in cord blood and maternal sera. Serum samples from 75 mothers (38 allergic, 37 non-allergic), and cord blood from their infants, were investigated for the presence of Fel d 1–IgG immune complexes (ICs) by using an amplified enzyme-linked immunosorbent assay (ELISA). Three monoclonal antibodies to Fel d 1 were used for coating. The specificity of the method was confirmed by inhibition experiments. ICs of Fel d 1–IgG were detected in the sera of 45% allergic and 49% non-allergic mothers, and in, respectively, 34% and 41% of their infants. Therefore, neither the prevalence nor the level of ICs were affected by maternal allergy. Low levels of trans-placentally transferred ICs can provide the fetus with a signal for the priming of T-cell responses to inhalant allergens. However, this is not necessarily related to allergic disease.

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The role of the post-natal exposure to allergens in the expression of atopy, and the question of when and how the T-cell priming to environmental allergens is initiated, have been discussed in recent years (1). It has been established that specific T-cell responses in new-born babies can be demonstrated in cord blood, even prior to any direct exposure after birth (2,3). Furthermore, T-cell proliferation to *Betula verrucosa* pollen extract has been reported in a 7-month-old child who had not yet experienced a birch pollen season (4). This cord blood mononuclear cell response to food and inhalant allergens has been reported to be common in infants both with and without a family history of atopy (3,5).

Some studies have reported the presence of immunoglobulin G (IgG) antibodies to inhalant allergens in virtually all neonatal blood samples,

both from atopic and non-atopic individuals (6,7), as well as a T-cell response to inhalant allergens in nearly 100% of blood samples from atopic and non-atopic individuals (8,9). In contrast, immune responses to antigens to which the pregnant women are not generally exposed have not been found (5).

It has been suggested that peptides processed by the mother might access the fetal circulation, either as free antigens or in complexes with IgG (10,11). We have recently reported the presence of the major cat allergen, Fel d 1, in sera from asthmatic children allergic to cat (12). The allergen was also found in complexes with IgG and immunoglobulin E (IgE) antibodies. Such complexes present in the maternal circulation could provide the fetus with the initial trigger for priming of the T-cell system already present *in utero*.

The objective of this work was therefore to investigate serum samples from allergic and non-allergic mothers, and cord blood from their babies, for the presence of Fel d 1 in immune complexes (ICs) with IgG antibodies.

Materials and methods

Study group

Serum samples were obtained from 38 allergic and 37 non-allergic mothers and their infants, based on the availability of both cord blood and sera. The families all participated in a prospective study related to the development of allergy in early childhood. Informed consent for participation in the study was obtained from the parents.

The samples were collected and stored at -20°C until analyzed. The allergic status of the mothers (e.g. allergic rhinoconjunctivitis, allergic asthma and dermatitis) was established from their clinical history, and the presence of circulating IgE antibodies to a panel of inhalant allergens was measured by using an Allergy Screen (Magic Lite SQ[™] Allergy System; ALK, Hørsholm, Denmark).

The children were followed-up until 18 months of age, and medical examinations for the diagnosis of atopic eczema were performed. The parents completed questionnaires regarding the presence of pets at home and exposure to pets outside the home, symptoms of allergy, and infections. Any reaction to food, which occurred at least twice, was recorded, and skin-prick testing to common allergens was performed according to the recommendations of the European Academy of Allergy and Clinical Immunology (13).

Antibody analyses

The levels of IgE antibodies to cat allergens in the sera from the mothers and their respective child at 18 months of age were determined by using a chemiluminescent immunoassay (Magic Lite[®]; ALK-Abelló). Levels higher than 1.43 standardized units (SU)/ml (class 1) were considered positive.

Circulating IgG antibodies to cat allergen in cord blood and in the maternal sera were measured by using a conventional enzyme-linked immunosorbent assay (ELISA), as previously described for the birch major allergen, Bet v 1 (7). Briefly, microtiter plates were coated with 200 μl /well of cat extract (ALK-Abelló) at 10 mg/l in phosphate-buffered saline (PBS). After blocking the protein-binding sites with 200 μl /well of human serum albumin (HSA), 0.5% in PBS-Tween, 200 μl of serum samples were added. Alkaline phosphatase-conjugated rabbit

anti-human IgG was then added and the reaction was developed by using FAST-*p*NPP (*p*-nitrophenyl phosphate) substrate (Sigma, St. Louis, MO, USA).

Immune complexes

An amplified ELISA was developed to increase the sensitivity of the assay. Costar 5390 microplates (Life Technologies AB, Täby, Sweden) were coated with the anti-Fel d 1 monoclonal antibody (mAb) in ascites, KAT 1 (kindly supplied by Carsten Schou; ALK), diluted in carbonate-bicarbonate buffer. To assess the specificity of the system, two HPLC-purified mAbs to Fel d 1, 3E4 and 6F9 (kindly supplied by Martin Chapman, University of Charlottesville, Virginia, VA, USA), were also used for coating. The plates were incubated overnight in a moist chamber at room temperature. After aspiration, the plates were washed four times in wash buffer (PBS-Tween, 0.05%). Further washing steps were included between the incubations until addition of the substrate. The free binding sites on the wells were blocked with 250 μl of 2% HSA in PBS at 37°C for 3 h, and then at 4°C overnight. Sera and cord blood samples diluted in PBS were added and then incubated for 1 h at 37°C . Duplicate wells containing PBS alone were used as blanks. Subsequently, 100 μl /well of A-3150 alkaline phosphatase-conjugated goat anti-human IgG (Sigma-Aldrich, Stockholm, Sweden), diluted 1:40 000 in AMPAK (Dakopatts AB, Älvsjö, Sweden) wash buffer and HSA 0.5%, was added. As substrate, AMPAK (Dakopatts) kits were employed. After washing with AMPAK buffer, the plates were incubated for 20 min with NADPH in diethanolamine buffer and for 10 min with the amplifier solution. The reaction was stopped by addition of 50 μl /well of 0.46 M sulfuric acid. The absorbance (*A*) was measured at 492 nm. A reference curve was prepared using a sample with Fel d 1-IgG IC, previously detected by purification and Western blotting. The levels of ICs in the samples were calculated from the *As* of the reference curve after subtracting the *A* of the blanks, and are expressed as arbitrary units (AU). Ratios higher than 2, corresponding to >30 AU/ml, were considered to be positive.

Samples from 20 children (7–17 years old), with ($n=11$) and without ($n=9$) detectable Fel d 1-IgG IC (12), were used as controls.

Any cross-reactivity between the conjugate and the mouse mAb was eliminated by incubation for 1 h at 37°C with a mouse serum at 10% (Lot 086; Dakopatts AB, Älvsjö, Sweden) prior to use.

Inhibition assay

The specificity of the method was confirmed by inhibition of the Fel d 1-IgG ICs with mAbs to cat. A serum sample with a high level of Fel d 1-IgG ICs was incubated for 1 h at 37°C with a pool of mAbs to Fel d 1, i.e. KAT 1, 3E4, and 6F9. The pool was prepared with 48 mg/l of 6F9 and 3E4, and 100 mg/l of KAT 1. The concentrated pool and a 10-fold dilution of the pool were used for incubation. As control, the same sample was incubated with two dilutions (140 and 70 mg/l) of 6D7, an HPLC-purified mAb to Der f 3, a major mite allergen (kindly supplied by Martin Chapman), and B v 09, a mAb in ascites to Bet v 1, a major birch allergen (kindly supplied by Carsten Schou). The samples were then analysed for ICs, as described above.

Statistical analysis

As the IC levels were not normally distributed, even after logarithmic transformation, groups were compared by using non-parametric tests. Comparisons between unpaired groups were performed by using the Mann-Whitney *U*-test, whereas paired groups were compared by using the Wilcoxon paired signed rank test. Fisher's exact test was used to compare proportions in unpaired groups, as the frequency for any cell was less than 5. MacNemar's test was used for the comparison of paired binary data. Calculations were made by using a statistical package, StatView 4.5 for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA).

Ethical aspects

The study was approved by the Regional Ethics Committee for Human Research, at the Linköping University Hospital, Sweden.

Results

Optimal conditions were established for the detection of Fel d 1-IgG ICs by ELISA. The specificity of the system previously demonstrated in the ELISA, using three different mAbs to cat allergen in the coating step, one in ascites, and two HPLC-purified mAbs, was assessed by ELISA inhibition (Fig. 1), which confirmed that the detection of ICs was not the result of any artefact inherent to the method.

The prevalence (Table 1) of ICs was similar in allergic and non-allergic mothers. Thus, Fel d 1-IgG ICs were detected in 45% of the allergic and 49% of the non-allergic mothers, whereas 34% of the cord blood from infants with allergic mothers

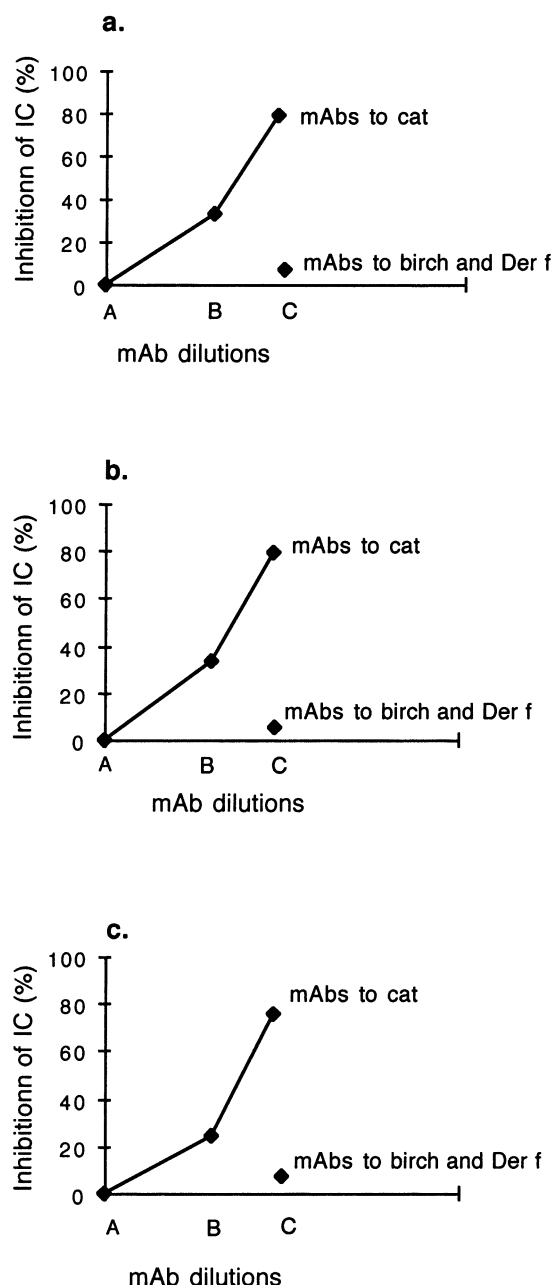


Fig. 1. Inhibition of enzyme-linked immunosorbent assay (ELISA) was employed, using three monoclonal antibodies (mAbs), for the detection of circulating Fel d 1-immunoglobulin G (IgG) immune complexes (ICs) in cord blood and sera. The plates were coated with: (a) KAT 1 mAb; (b) 3E4 mAb; and (c) 6F9 mAb. The mAb dilutions correspond to: (A) buffer only; (B) a 10-fold dilution; and (C) a concentrated pool of mAbs. For mAbs to birch and Der f, the dilutions were: (A) buffer only; (B) 70 mg/l; and (C) 140 mg/l of both antibodies.

and 41% of cord blood from infants with non-allergic mothers had detectable levels of Fel d 1-IgG ICs. Similar levels (Fig. 2) of ICs were detected in allergic and non-allergic mothers ($p=0.45$, CI=59–247, mean 193), as well as in the cord blood of their babies ($p=0.38$, CI=16–232, mean 183) (Mann-Whitney *U*-test).

Table 1. Number of samples (n) with detectable Fel d 1-immunoglobulin G (IgG) immune complexes (ICs) in cord blood and serum from allergic and non-allergic mothers

	Allergic, n=38		Non-allergic, n=37	
	Cord blood	Maternal serum	Cord blood	Maternal serum
Samples with ICs	13	17	15	18
Cat at home	5	6	6	5
Exposure weekly	6	7	5	5
Non-exposed	2	4	4	8

The detection of ICs is shown in relation to the presence of a cat at home, direct exposure to cat at least once a week, and no known regular exposure. n=number of samples.

There was a relationship between the presence of ICs in cord blood and maternal serum in the allergic ($p=0.63$) and in the non-allergic ($p=0.29$) groups (MacNemar's Test). The levels of ICs in cord blood were similar to those in the corresponding maternal sera, in both the atopic ($p=0.75$, CI=60–292, mean 203) and the non-atopic ($p=0.93$, CI=40–244, mean 179) groups (Wilcoxon paired sign test). ICs were detected, both in cord blood of infants and in the sera of their mothers, in 32% and 27% of the allergic and non-allergic groups, respectively. Only in three of 28 of the positive cord blood samples were ICs not detectable in the corresponding maternal serum.

IgG antibodies to cat were detected in 11/17 allergic mothers and in eight of 18 non-allergic mothers with detectable ICs, and the levels were similar in the two groups. Specific IgE antibodies to cat were detected in nine allergic mothers and three of their children, as compared to none of the non-allergic mothers and three of their infants. There was no relationship between the presence of IgE and IgG antibodies to cat ($p=0.01$) or

between IgE antibodies and ICs in the sera ($p=0.003$, Fisher's exact test).

The presence of ICs correlated with the exposure to cat (Table 2). This was particularly true in the maternal sera (no statistical analysis could be performed in the allergic mothers, as all the samples had detectable ICs). Even for this group, however, the presence of ICs was related to exposure to cat, as the number of cat-exposed mothers with detectable ICs was even higher than the non-allergic mothers. Furthermore, these highest levels were observed in exposed individuals. Thus, ICs were detected in four of eight cat-allergic mothers and their infants. However, direct exposure was not always associated with the presence of ICs. ICs were not detectable in sera from three allergic and five non-allergic mothers, and their respective children, who had a cat at home. In only two of those eight mothers (both non-allergic), were cat IgG antibodies undetectable.

Fourteen of the 38 children with allergic mothers developed allergic symptoms during the first 18 months of life, and eight of them had detectable ICs in cord blood. The finding was similar in children of non-allergic mothers, i.e. 16/37 children developed allergic symptoms, and ICs were present in the cord blood in nine. The six children with the highest levels of ICs in cord blood, three with allergic and three with non-allergic mothers, developed allergic symptoms.

Discussion

Immune responses to inhalant allergens are commonly detected in cord blood cells in both atopic and non-atopic individuals (4,14). These responses are of fetal origin, as proven by the DNA extracted from fetal T-cell clones (15). The presence of the major allergen, Fel d 1, in cord blood supports previous suggestions that the specific T-cell response could be a result of fetal exposure to low levels of allergenic peptides (10,15,16) either as free antigens or in the form of ICs with maternal IgG antibodies commonly

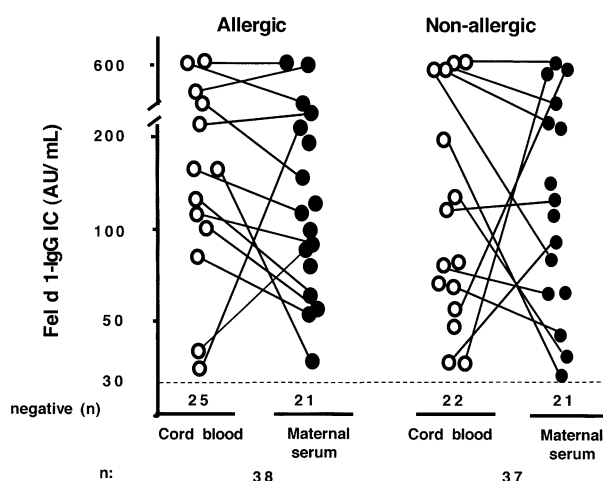


Fig. 2. Levels of immune complexes (ICs) in sera (closed circles) from allergic and non-allergic mothers and in cord blood (open circles) from their babies. Samples from mother-infant pairs are united by a line. The levels are expressed in arbitrary units (AU). The detection level was 30 AU/ml.

Table 2. Correlation between the exposure to cat and the detection of immune complexes (ICs) in cord blood and sera from allergic and non-allergic mothers

	Allergic			Non-allergic		
	Exposed	Non-exposed	p-value	Exposed	Non-exposed	p-value
Cord blood						
Yes	10	2	p=0.54	11	2	p=0.26
No	3	2		3	3	
Maternal serum						
Yes	13	4	p-value*	12	5	p=1.0
No	0	0		2	0	

The values represent the number of samples with detectable (yes) and non-detectable (no) ICs.

The p-values indicate differences among the groups (Fisher's Exact Test).

present in cord blood (7). Our finding of inhalant allergens in cord blood in complexes with IgG could therefore explain how fetal T-cell responses to inhalant allergens would be induced in neonates. The findings do not exclude other mechanisms for T-cell stimulation. It is not known, however, how such fetal immune responses to allergens affect post-natal immunity, i.e. whether or not they are associated with allergy development. The lack of any relationship to allergy in our study would indicate that early immune responses are not necessarily associated with disease and may in fact be part of the normal development of immunity.

The detection of ICs in both allergic and non-allergic individuals may appear to conflict with results of a previous study, in which ICs were only found in older allergic children and not in those who were non-allergic (12). In that study, however, the control sera were selected from healthy individuals with no or very low levels of IgG, while in the present study most samples contained IgG antibodies to cat.

The biological significance of ICs is controversial. It has been reported that they may enhance the function of antigen-presenting cells (17). It has also been reported that Der p 2-ICs stimulate the proliferation of specific T cells at a 1000-fold lower Der p 2 concentration than required for T-cell activation by native antigen (18). Therefore, ICs containing inhalant allergens may facilitate the development of immunity. This, however, may not necessarily be associated with the development of allergy.

The detection of ICs in sera from mothers without any known contact with cats is explained by the ubiquitous presence of cat allergen in Sweden, even in places where cats are not allowed (19,20). On the other hand, the lack of a clear association between exposure to cat and the presence of ICs suggests that the presence of complexes was not only related to the amount of exposure.

Furthermore, the ELISA employed in the detection of ICs was up to 30 times more sensitive

than the standard ELISA used to detect IgG antibodies to cat. This would explain why ICs were detected in some samples with undetectable levels of IgG antibodies to cat.

It has been proposed that mothers with a high IgG response to a certain allergen might also circulate high levels of the corresponding antigen more effectively (10). Our results indicate that the levels of IgG in the maternal sera were not directly related with the presence and/or levels of ICs in cord blood.

The presence of ICs in cord blood, independently of the allergic status of the mother, could provide a possible mechanism for the previously established immune response to inhalant allergens in cord blood, and suggests that small populations of allergen-specific T cells in the newborn can be primed during intra-uterine life as part of the normal maturation of the immune system in the fetus (21), as virtually all infants are born with their immune systems primed for the development of immunological memory against environmental allergens (22).

In conclusion, low levels of trans-placentally transferred allergens, in the form of ICs with maternally derived IgG antibodies, could provide the initial signal for T-cell priming to inhalant allergens already present in the fetus. This seems to be the case, in both allergic and non-allergic individuals, suggesting that it may represent a normal stimulatory signal, which is not necessarily associated with sensitization and future allergic disease. After birth, environmental factors will influence the T-helper 2 (Th2) response, skewed to deviate towards a balanced Th1/Th2-type of immunity, or a prolonged Th2-skewed response associated with allergy.

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