

Evolution of IgM, IgE and IgG_{1–4} antibody responses in early childhood monitored with recombinant allergen components: implications for class switch mechanisms

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The formation of IgE antibodies against environmental allergens represents the hallmark of type I allergy. Data from *in vitro* cultured cells and experimental animal models provide controversial evidence for isotype switching from IgM to IgE production via sequential as well as non-sequential (*i.e.* direct) class switch. We analyzed the evolution of IgE responses in 11 children developing birch pollen and/or grass pollen allergy during the first 7 years of life using purified recombinant allergen molecules (major birch pollen allergen, Bet v 1; major timothy grass pollen allergens, Phl p 1, Phl p 2, Phl p 5). Demographic, clinical and serological data indicated a postnatal sensitization to pollen allergens. A parallel development of IgG_{1–4} and IgE responses to recombinant allergen molecules compatible with a strictly sequential class switch to IgE was observed only in one child. The only partly synchronized and dissociated development of allergen-specific antibody responses found in all other cases can be best explained by a partly sequential class switch involving few switch stations or, more likely, by direct class switching. Kinetics and courses of allergen-specific antibody responses (IgM, IgG_{1–4}, IgE) during the first years of life suggest that, once established, allergen-specific IgE responses are driven by antigen contact rather than by cytokines controlling class switch to IgE.

Key words: Type I allergy / IgE / Isotype switch / Recombinant pollen allergen

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1 Introduction

Type I allergy, an immunoglobulin E (IgE)-mediated hypersensitivity disease with complex genetic background affects almost 25% of the population [1]. Allergic individuals can be discriminated from non-atopic persons by their increased tendency to produce IgE antibodies against *per se* harmless antigens (*i.e.* allergens) which are presented to the immune system mainly via the respiratory or gastrointestinal tract [2]. In sensitized individuals, allergen contact can elicit a plethora of allergic symptoms (*e.g.* allergic rhinoconjunctivitis, asthma, dermatitis, food allergy, anaphylactic shock). Allergen-induced cross-linking of effector cell (*e.g.* mast cell,

basophil) -bound IgE induces the liberation of preformed (*e.g.* histamine) and *de novo* synthesized (*e.g.* leukotrienes) inflammatory mediators [3, 4]. Moreover, IgE can enhance allergic immune responses by facilitating allergen capture and presentation by B cells via the low-affinity IgE receptor, CD23, and by professional antigen-presenting cells (monocytes, dendritic cells) via the high-affinity receptor, FcεRI, to T cells [5–8]. IgE apparently can also perpetuate the disease by its ability to up-regulate the expression of its own receptors, FcεRI and CD23 [9].

The formation of IgE antibodies requires immunoglobulin class switching from IgM- to IgE-producing B cells, an event that is stimulated by contact with minute antigen doses, cytokines (IL-4 and IL-13) and CD40 activation (reviewed in [10]). In principle, switching to IgE production may occur directly from IgM to IgE without

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intermediate stages or sequentially, such that a B cell that had previously switched to, e.g. C γ 1 can later switch to an isotype located 3' to the C γ 1 gene, e.g. C α 1 or C ϵ [11, 12].

Whether switching to IgE production in allergic patients occurs directly or sequentially represents a controversial issue that has been indirectly approached by *in vitro* studies and via experimental animal models. While on the one hand it has been shown in mice that switching from IgM to IgE often proceeds sequentially via IgG₁ [13, 14], it was also shown that in mutant mice, in which sequential switching to IgE via IgG₁ was blocked, the frequency of cells switching to IgE was not affected [15]. Polymerase chain reaction (PCR)-amplified switch circles isolated from cultured human B cells after stimulation with IL-4 [16–19] as well as germ-line transcripts from patients with *Schistosoma mansoni* [20] provided evidence for direct μ to ϵ , sequential μ - γ - ϵ as well as double-sequential μ - γ - α - ϵ or μ - γ 1- γ 2- ϵ switching. Moreover, the analysis of S μ /S ϵ switch regions from freshly isolated B lymphocytes from atopic patients argued for a predominantly direct IgM to IgE class switch program [21].

To obtain information about the *in vivo* evolution of IgE responses in allergic individuals we have collected

serum samples from children developing birch and/or grass pollen allergy during the first 7 years of life. These serum samples were analyzed for the development of allergen-specific IgM, IgG₁₋₄ and IgE antibodies to purified recombinant allergen molecules (major birch pollen allergen, Bet v 1; major timothy grass pollen allergens, Phl p 1, Phl p 2, Phl p 5). Using purified recombinant allergen molecules we intended to determine the development, kinetics and courses of IgM, IgG₁₋₄ and IgE antibody responses with specificity for four of the most important respiratory allergens and to correlate these parameters with the development of clinical sensitization. Our findings support the concept of an early post-natal sensitization accompanied either by a partly sequential or more likely, by a direct class switch to IgE production which, once established, is driven mainly by antigen contact.

2 Results

2.1 Demographic, clinical and serological characterization of the allergic children: clinical evolution of allergic sensitization

Table 1 summarizes the demographic and clinical data of the eight boys and three girls whose sera were analyzed

Table 1. Demographic, clinical and serological data of atopic children^{a)}

	Date of birth	Sex	Atopic parents	Development of			Total IGE kU/l	specific IgE					
				Rhinitis and conjunctivitis	Asthma	Atopic dermatitis		Birch kUAl/l	Bet v 1 OD	Timothy kUA/l	Phl p1 OD	Phl p 2 OD	Phl p 5 OD
1	1/1/90	M	1	5y; 7y	2y; 4–5y	–	182	–	–	6.42	0.12	–	–
2	11/16/90	M	1	5–6y	1y	3y	262	38.70	0.22	21.70	0.22	–	0.10
3	12/13/90	F	1	6–7y	–	–	132	83.90	0.33	–	–	–	–
4	10/30/90	M	0	4y; 6–7y	–	–	331	2.82	–	80.20	0.20	0.14	0.22
5	11/29/90	M	0	1–5y	–	3m–3y, 6y–7y	1601	100	0.32	41.7	0.26	–	–
6	10/7/90	F	1	7y	–	–	63	4.42	–	16.4	0.07	–	0.07
7	10/2/90	M	1	5–7y	–	–	49.2	–	–	10.0	–	–	0.08
8	12/27/90	F	1	–	3–6y	–	841	–	–	3.73	0.07	–	–
9	1/16/90	M	0	–	3m; 6y	–	42.7	–	–	1.66	–	–	–
10	11/15/90	M	2	5–7y	3–7y	1y–7y	336	–	–	10.40	0.08	–	–
11	9/23/90	M	1	5–7y	6y	–	330	–	–	98.60	0.35	0.11	0.24

a) Date of birth, sex, family history of atopy, development of allergic symptoms and serological data obtained at the age of seven are displayed. Dashes indicate negative results (M: male, F: female; y: year, m: month).

in this study. Eight of the children had at least one atopic parent. All children were born during autumn and winter (23.09.–01.01.), a period outside the flowering period of birch (February–April) and grasses (May–July). The latter fact argues against the possibility of an intrauterine sensitization of the children to birch or grass pollen allergens because of the immature stage of the fetal immune system during the possible periods of maternal pollen allergen encounter [22–24].

Symptoms of allergy developed in the children between 3 month to 7 years and comprised seasonal allergic rhinoconjunctivitis (9/11 children), asthma (6/11 children) and atopic dermatitis (3/11 children). Total IgE levels as determined at the age of 7 years ranged between 42.7 and 1601 kU/l. The sensitization to birch pollen allergens was confirmed by serological measurement of IgE antibodies to natural birch pollen extract or rBet v 1 in five children at the age of 7 years. Ten children exhibited elevated IgE antibody levels to natural and/or recombinant grass pollen allergens at the age of 7 years. All children with specific IgE antibodies exhibited immediate skin reactions when tested with birch and grass pollen allergen extracts (data not shown).

2.2 Evolution of IgE responses to allergen extracts and individual allergen components

The recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) that were used to analyze the evolution of IgE responses in the allergic children represent markers for genuine grass pollen sensitization [25, 26]. They lack cross-reactivity with allergens from plant species other than monocotyledonic plants (grasses, corn) and are only expressed in pollen but not in other plant tissues (e.g. leaves, seeds, roots). While rPhl p 1 shares cross-reactive IgE epitopes with group 1 allergens of most monocotyledonic plants (*Panicoideae*, *Pooideae*), rPhl p 2 and rPhl p 5-related allergens occur mainly in *Pooideae* but are not present in *Panicoideae* [25].

As exemplified in Fig. 1A (patient 4), we observed in most of the grass pollen-allergic children a parallel increase of IgE antibodies to the different recombinant grass pollen allergens and to timothy grass pollen extract. Interestingly, one child (patient 5; Table 1) showed a dissociated evolution of IgE responses to the individual recombinant grass pollen allergens (Fig. 1B). In this child IgE antibodies to all three allergens were already detectable at the age of 2 years. Until year 7, rPhl p 1-specific IgE levels increased strongly, whereas rPhl p 2 and rPhl p 5-specific IgE antibodies decreased continuously, a fact that might be explained by a preferential exposure to *Panicoideae* pollen in this period of life.

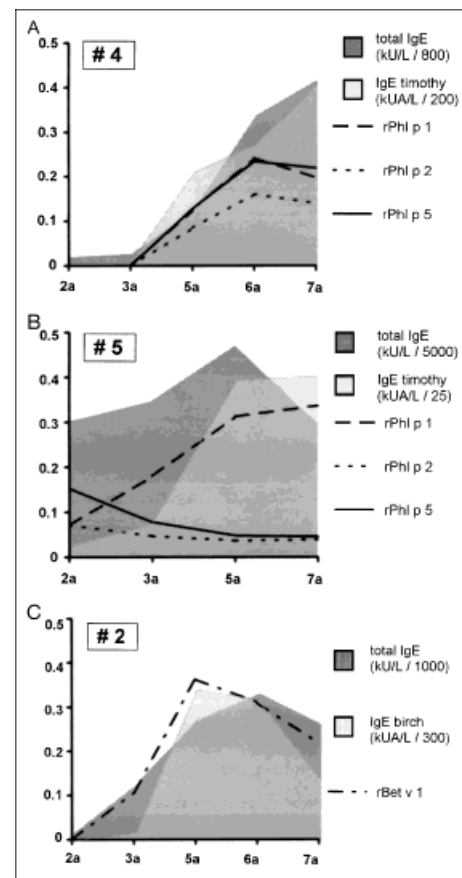


Fig. 1. Evolution of IgE responses monitored with allergen extracts and recombinant allergens. Total serum IgE levels (kU/l), specific IgE responses to recombinant allergens (OD values) and pollen extracts (timothy grass, birch) (kU/l) are displayed on the y-axis. The annotations on the x-axis specify the ages (a: year) of the children when serum samples were obtained. (A, B) IgE responses to recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) for two children (patients 4 and 5). In (C) the evolution of the IgE response to recombinant Bet v 1, the major birch pollen allergen is displayed.

Sensitization to birch pollen was monitored using recombinant Bet v 1, the major birch pollen allergen [27]. Bet v 1 cross-reacts with the major allergens of early flowering trees of the order *Fagales* (e.g. alder, hazel, hornbeam, oak), with allergens in plant food (fruits, vegetables, spices) but not with pollen allergens from other plants (e.g. grasses, weeds) [27, 28]. As far as crossreactivity with plant food allergens is concerned, there is strong evidence that respiratory sensitization to pollen-derived Bet v 1 drives the cross-reactive immune response to the homologs in plant food [29]. As shown in Fig. 1C (patient 2), IgE responses to rBet v 1 were strictly in parallel to those against birch pollen extract in birch pollen-allergic children, a finding that may be explained by the fact that

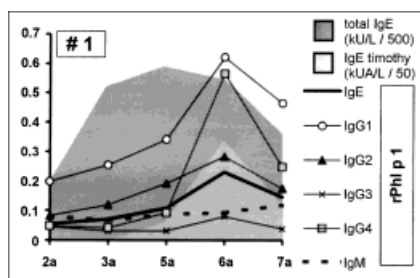


Fig. 2

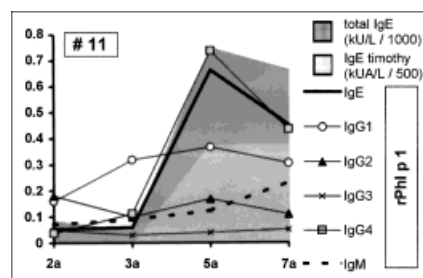


Fig. 6

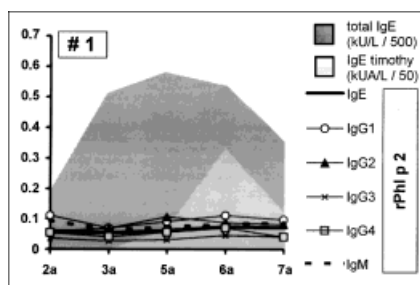


Fig. 3

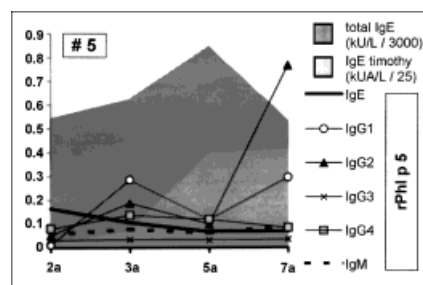


Fig. 7

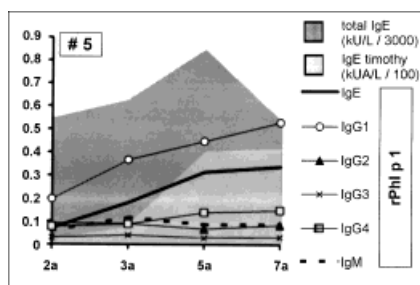


Fig. 4

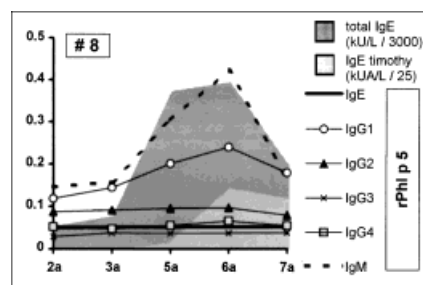


Fig. 8

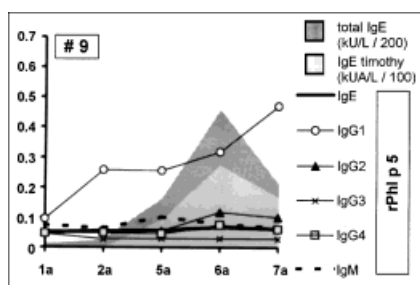


Fig. 5

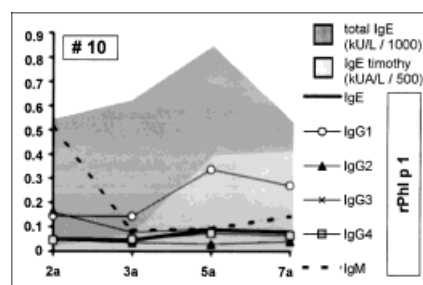


Fig. 9

Fig. 2–9. Evolution of IgE, IgM and IgG subclass reactivity to recombinant timothy grass pollen allergens. Total serum IgE levels (kU/l), specific IgE, IgG₁₋₄ and IgM responses to recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) (OD values) and timothy grass pollen extract-specific IgE levels (kU/l) are displayed on the y-axis. The annotations on the x-axis specify the ages (a: year) of the children when serum samples were obtained. Patients numbers and recombinant allergens are indicated in each figure.

Bet v 1 represents the major allergenic component of birch pollen.

As exemplified for a child (patient 4) with an exclusive grass pollen sensitization total IgE responses were sometimes in parallel to allergen-specific IgE levels (Fig. 1A), whereas in other children (e. g., patient 5), with a sensitization to many different allergen sources (Fig. 1B), different kinetics were observed. Using either pollen extracts (timothy grass, birch) or recombinant pollen allergens, specific IgE antibodies could be detected after 1 year in one child, after 2 years in three children, after 3 years in three children and after 5 years in the remaining four children (Table 1). As shown in Fig. 1B, established allergen-specific IgE levels did not further increase between the 5th and 7th year in four children (patients 5, 9, 10, 11) and in two cases (e.g. patient 1: timothy grass; patient 2: birch, rBet v 1) even decreased in the 7th year (Fig. 1C; data not shown). As exemplified for patient 4, a continuous and strong increase of specific IgE responses was noted for the other children (patients 3, 4, 6, 7, 8) between the ages of 5 and 7 years (Fig. 1A).

2.3 Monitoring of IgM, IgG₁₋₄ and IgE responses to defined recombinant allergens in early childhood: implications for *in vivo* class switching

2.3.1 Examples for the evolution of allergen-specific antibody responses in agreement with a "strictly (double) sequential" class switch

When we used recombinant birch and timothy grass pollen allergens to monitor the development of IgM, IgG₁₋₄ and IgE responses during the first 7 years of life, we observed different antibody reactivity profiles and kinetics; of these we describe representative examples and their possible implications for class switch mechanisms in detail.

In the case of a strictly sequential (*i.e.* double-sequential) isotype switch, it would be expected that B cells produce IgM, IgG₃, IgG₁, IgG₂, IgG₄ and IgE with specificity for a given allergen and that all of these antibody classes/subclasses can be detected. Among all 11 children we found only one in whom IgM, IgG₁₋₄ and IgE against a defined allergen (rPhl p 1) developed simultaneously and showed parallel kinetics (Fig. 2: patient 1). In this patient no specific antibody responses could be detected to another grass pollen allergen, rPhl p 2 (Fig. 3). During the first 7 years no specific IgE antibodies to rPhl p 5 could be detected in this patient, but rPhl p 5-specific IgM and

IgG antibodies started to develop after year 3 (data not shown).

2.3.2 Partly synchronized development of allergen-specific IgE and IgG subclass responses due to either a partly sequential or a non-sequential class-switch

In most patients we found that several, but not all, antibody classes or subclasses to a given allergen component developed simultaneously and followed parallel kinetics during the first 7 years of life. In certain patients (e.g. patient 5, rPhl p 1) we detected parallel increases of IgE, IgG₁ and IgG₄ antibody responses (Fig. 4). Certain individuals showed a parallel development of IgG subclass responses (e.g. patient 9, rPhl p 5: IgG₁, IgG₂) without an accompanying IgE production (Fig. 5). The parallel development of allergen-specific antibody responses in certain immunoglobulin classes and subclasses observed in the latter patients could be explained either by a partly sequential or a non-sequential class switch.

2.3.3 Dissociated and anti-parallel development of allergen-specific IgM, IgE and IgG subclass responses in accordance with non-sequential class switch

Fig. 6 shows an example of a dissociated and antiparallel development of allergen-specific IgE, IgM and IgG subclass responses (patient 11, rPhl p 1) as was observed in most children. Allergen-specific IgG₁ and IgG₂ levels were detectable already at the age of 2 years. While IgG₁ increased until the age of 5, IgG₂ decreased at the age of 3 and increased at the age of 5 years again. In contrast to IgG₁ and IgG₂, allergen-specific IgG₄ and IgE levels were not detectable until the age of 3 and showed a rapid increase at the age of 5 years. Interestingly, allergen-specific IgM levels were low between the age of 2–5 years, when IgE and IgG subclass responses were already established, but started to increase at the age of 7 years.

In patient 5, rPhl p 5-specific IgE was already elevated at the age of 2, but decreased continuously until the age of 7 years (Fig. 7). Allergen-specific IgG₂ showed a slight and a strong increase at the ages of 3 and 7, respectively. Also allergen-specific IgG₁ showed increases at the age of 3 and 7 years whereas IgM, IgG₃ and IgG₄ remained low throughout the first 7 years of life. The different orders and kinetics of antibody class and subclass increases (patient 11: IgG₁, IgG₂, IgG₄ and IgE before IgM; patient 5: IgE before IgG₁ and IgG₂) argue against a sequential class switch in these patients.

2.4 Association of allergen-specific IgM with IgE and IgG subclass responses

In most children we could not detect significantly elevated levels of allergen-specific IgM responses prior to the development of allergen-specific IgG subclass and IgE responses. In a few children (e.g. patient 8, rPhl p 5) we found a parallel rise of allergen-specific IgM and IgG subclass responses during the period of monitoring (years 2–7) (Fig. 8). Certain children exhibited strongly elevated allergen-specific IgM responses at the beginning of the monitoring period (patient 10, rPhl p 1, year 2), which decreased strongly during years 3–7 (Fig. 9). While in the latter patient IgG₁ responses developed subsequently between years 3 and 7, no other IgG subclasses or IgE responses occurred until the age of 7. The latter two examples show that allergen-specific IgM responses can be detected at different periods of life but do not necessarily lead to formation of IgE or IgG subclass responses.

3 Discussion

We have retrospectively studied children acquiring respiratory allergy to birch and/or grass pollen allergens during the first 7 years of life to obtain information regarding the development of specific IgE, IgM and IgG_{1–4} antibody responses. Instead of using allergen extracts which consist of undefined mixtures of allergenic and non-allergenic molecules, we studied the development and kinetics of antibody responses to major birch and timothy grass pollen allergens produced as recombinant allergen molecules [30]. We were thus able to determine the evolution of antibody responses with specificity for defined allergens during and after the natural sensitization phase in allergic children. In all but one child, IgE antibodies with specificity to respiratory pollen allergens were not detectable before the age of 2 years. The fact that all children were born either in autumn or winter makes it unlikely that they were sensitized *in utero* because of the immature stage of their immune system during the flowering season of birch (spring) and grasses (May–July). The latter consideration together with the kinetics of IgE responses (*i.e.* development mainly during and shortly after the 2nd year of age) indicate a postnatal sensitization most likely during the 1st year of life, when the children experienced their first birch and grass pollen contact. The continuous rise of allergen-specific IgE levels after the proposed sensitization event is likely due to repeated seasonal allergen contact, which is known to induce considerable rises of IgE levels also in sensitized adults [31]. Development, course and kinetics of allergen-specific IgM, IgG_{1–4} and IgE responses were dissociated, and thus indicated either a partly sequential

class switch involving few switch stations or, more likely, a non-sequential (*i.e.* direct) class switching to IgE or to certain IgG subclasses (Fig. 10). Only one child showed a parallel development of allergen-specific IgM, IgG_{1–4} and IgE responses compatible with a strictly sequential (*i.e.* double sequential) class switch model (Fig. 10). We are aware that on the basis of our data we cannot exclude the possibility that B cells representing intermediate stages of a strictly sequential class switch program were either not active, already deleted or produced too low amounts of antibodies at the time points of serum sampling. On the other hand we frequently observed that the production of allergen-specific IgE preceded the production of allergen-specific IgG_{1–4} and, in certain cases, also the production of allergen-specific IgM, a finding which is best explained by a non-sequential (*i.e.* direct) class switch.

In most children allergen-specific IgM could not be detected prior to formation of allergen-specific IgG_{1–4} and IgE and, in certain cases, a strong induction of allergen-specific IgM levels was observed without consecutive development of IgG_{1–4} or IgE responses. We consider this finding interesting in view of a recent report describing the formation of specific IgA antibodies in μ MT mice which have a pro-B cell developmental block and lack IgM or IgD expression [32]. This report, together with the observation of local IgE production in the nasal mucosa of atopics [33], leaves room for speculations regarding the possibility of a direct transition to IgE production in the target organs of atopy, a scenario which is in agreement with the finding that pro-B cells from recombination-activating gene 2-deficient (RAG-2^{-/-}) mice switch their isotype to C ϵ without expression of either the pre-BCR complex or mIgM [34].

The idea that a direct isotype switch to IgE during the sensitization event may be possible is highly speculative. However, our data strongly suggest that the once established IgE response is not linked to an ongoing IgM or IgG_{1–4} production, which is in agreement with early work identifying long-lived IgE-producing lymphocytes in the peripheral blood of allergic patients [35]. The latter finding together with the observation that successful immunotherapy is not paralleled by a decrease in IgE levels [36] question the role of IgE in the maintenance of allergic disease. On the other hand it has been demonstrated that the established IgE response is mainly antigen-driven [31]. Support for the view that this response does not stem from cytokine-induced *de novo* class switching of allergen-specific IgM-bearing cells to IgE comes from the observation that blood-derived IgE-secreting cells from allergic individuals exhibited spontaneous production of allergen-specific IgE antibodies and were not sensitive to IL-4, anti-IL-4, IL-13 or anti-IL-13 treatment [37, 38].

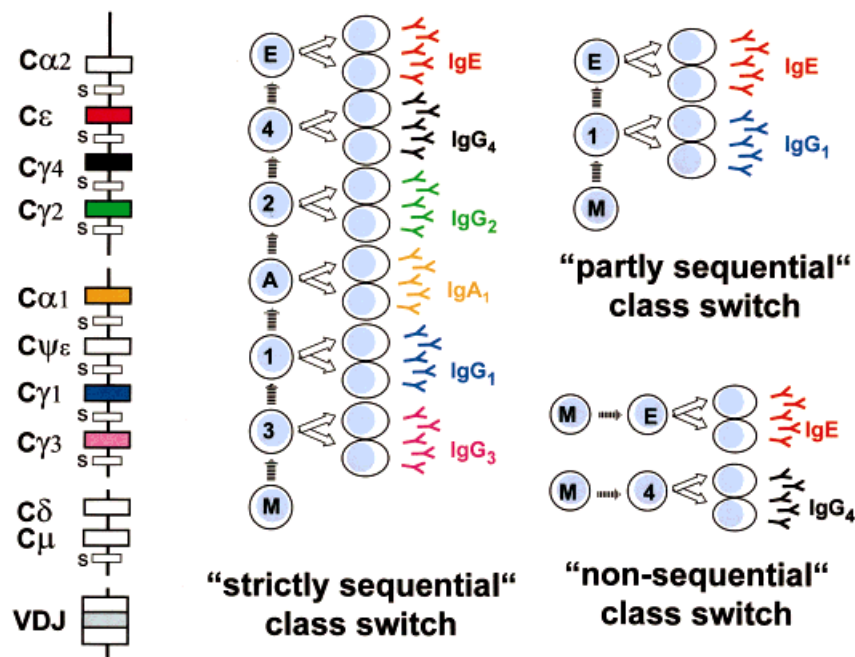


Fig. 10. Models for class switch compatible with the evolution of antibody responses found in the children. The left panel shows the schematic arrangement of immunoglobulin heavy chain genes in the human germ line. It consists of the VDJ locus, encoding for the antigen-binding domain of the immunoglobulin molecule, and genes encoding for the constant portion of the immunoglobulin heavy chains (C). Each C gene, except C δ , is flanked on its 5' side by a switch region (s), which can recombine with another s-region by deletional recombination. According to a strictly sequential class switch, allergen-specific IgM cells would sequentially, via IgG₃-, IgG₁-, IgA-, IgG₂- and IgG₄-producing cells switch to IgE-secreting cells with specificity for the same allergen. A partly sequential class switch would involve only certain stages (e.g. IgG₁) between the transition from IgM to IgE production. Alternatively different IgM precursors may switch directly to IgE or IgG subclass (e.g. IgG₄)-producing cells.

4 Patients and methods

4.1 Characterization allergic children

Sera from 11 children who developed birch pollen ($n=5$) and grass pollen ($n=10$) allergy were derived from the Multicenter Allergy Study (MAS) [39, 40]. In this cohort study, 1314 newborns from five German cities were recruited and monitored prospectively for 7 years regarding the development of allergy. The 11 children investigated in our study were born in autumn or winter, outside the flowering season of birch and grasses. The development of clinical symptoms were followed up at ages 3, 6, 12 and 18 months and annually until the 7th year of age. Blood samples were obtained at the age of 1, 2, 3, 5, 6 and 7 years, respectively.

4.2 Determination of total IgE, birch and timothy grass pollen-specific IgE levels

Total IgE and specific IgE levels to birch and timothy grass pollen extract were determined by quantitative CAP-RAST-FEIA (Pharmacia, Uppsala, Sweden) measurements annu-

ally. The serological diagnosis of allergy was confirmed by skin prick testing using natural birch and timothy grass pollen extracts (ALK, Horsholm, Denmark) at the age of 7 years. Reactions were read at 15 min and the SPT was considered as positive if the wheal diameter was >3 mm without reaction to sodium chloride.

4.3 ELISA determination of specific IgE, IgG₁₋₄ and IgM levels to recombinant pollen allergens

Recombinant timothy grass pollen allergens (rPhl p 1, rPhl p 2, rPhl p 5) and recombinant Bet v 1, the major birch pollen allergen, were expressed in *Escherichia coli* and purified to homogeneity as described [25, 41]. Each of the recombinant allergens was dissolved separately in 0.1 M calcium carbonate buffer, pH 9.6, at a concentration of 5 μ g/ml and coated to ELISA plates (Nunc Maxisorb, Roskilde, Denmark) by incubation at 4°C overnight. Plates were washed three times with buffer A (TBS, 0.05% vol/vol Tween 20) and blocked with buffer A containing 1% wt/vol BSA at room temperature for 4 h. ELISA plate-bound allergens were exposed to 100 μ L/well of each serum diluted in buffer A (1:10 for IgE;

1:20 for IgM; 1:100 for IgG₁₋₄) at 4°C overnight and washed five times with 200 µl of buffer A the next day. The isotype specificity of mouse monoclonal anti-human IgE (PharMingen, San Diego, CA), anti-human IgM (Sigma, St. Louis, MO) and anti-human IgG₁₋₄ antibodies (PharMingen) was confirmed by testing each of the antibodies for their reactivity to purified human IgE, IgM, IgA, IgG₁, IgG₂, IgG₃ and IgG₄ by ELISA as described previously [42]. Bound human IgE and IgM antibodies were detected with an alkaline phosphatase-coupled mouse monoclonal anti-human IgE antibody (PharMingen) and an alkaline phosphatase-coupled monoclonal anti-human IgM antibody (Sigma), respectively, as described [43]. Bound IgG₁₋₄ antibodies were detected with monoclonal mouse anti-human IgG₁, IgG₂, IgG₃ and IgG₄ antibodies (PharMingen), respectively, and a horseradish peroxidase-coupled sheep anti-mouse antiserum (Amersham, Little Chalfont, GB) as described [42]. The optical densities corresponding to the amount of bound antibodies were determined with an ELISA reader (Dynatech, Denkerdorf, Germany). To avoid plate-to-plate variabilities, all sera were tested on the same plate for each allergen and antibody class/subclass. Negative sera were included on each plate to establish a cut-off base line. Determinations were carried out in duplicates and results are displayed as mean values with a SD of less than 5%.

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