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Immune responses to birch in young children during their first seven years of life

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Running title: Birch induced immune responses

Abstract

Background: The character of immune responses to allergens during the first years of life may decide whether the individual will become tolerant or develop allergy later in life.

Objective: To study the development of immune responses to the seasonal inhalant allergen birch over the first seven years of life.

Methods: Blood samples were obtained from 21 children who were followed prospectively from the second to the seventh pollen season of life. Birch induced cytokine production and IgG subclass antibodies to rBet v 1 were analysed with ELISA, mRNA expression with real time PCR, IgE antibodies to birch with Magic Lite™ and birch induced mononuclear cell proliferation with ³H-thymidine incorporation.

Results: Birch induced IFN- γ and IL-10 production increased with age, both in atopic and non-atopic children, while birch induced IL-13 production decreased. The two children who were sensitised and developed clinical allergy to birch showed persistent IL-4 and IL-5 production and IL-9 mRNA expression, as well as Th2 associated IgG4 responses. Transient Th2-like responses were observed among the other children. Proliferative responses and IgG1 antibodies were seen in all children.

Conclusions: Immune responses to birch can be demonstrated in all children, during the first seven years of life, regardless of atopic status. A transient early Th2-like response is down-regulated after the fourth pollen season, except in children who develop clinical allergy to the particular allergen.

Key words: T cell responses, IgG subclass antibodies, birch, atopy, childhood

Abbreviations

AU	Arbitrary units
CBMC	Cord blood mononuclear cell
Cpm	Counts per minute
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
Th	T helper

Introduction

Allergic diseases are believed to be due to Th2-like immunity to allergens in affected tissue, and immune responses to allergens are characterised by a cross-regulation between Th1 and Th2 cells [1]. Th2 cells produce IgE synthesis inducing IL-4 [2], eosinophilia promoting IL-5 and IL-9 [3, 4], while IFN- γ from Th1 cells down-regulates IgE synthesis [2]. Interleukin-9 also enhances IgE production [5] and stimulates T cell and mast cell growth [6, 7]. Interleukin-10 is an anti-inflammatory cytokine, which inhibits both Th1 and Th2 cells and IgE production [8], as well as shortens eosinophil survival [9]. Less is known about Th1/Th2 development in atopic children.

Prospective studies have shown that IgE antibodies to inhalant allergens are commonly detected both in atopic and non-atopic infants during the first years of life [10]. In non-atopic children, the IgE responses to inhalant allergens are eventually down-regulated, whereas they reach higher levels and tend to persist in atopic children. Furthermore, IgG responses, primarily of the IgG1 subclass to inhalant allergens are also common in infancy, while high levels of IgG₄ subclass antibodies to allergens are associated with sensitisation and allergic symptoms [11]. The differences in IgG₄ antibody levels to birch in atopic

and non-atopic children increase from 18 months to 8 years, largely due to a down-regulation of IgG₄ antibody responses in the non-atopic children [11].

It has been demonstrated that birch induced IFN- γ is associated with IgG₁ antibody production, while IL-4 production relates to IgG₄ antibody subclass responses to Bet v1 [12]. The balance between Th1 and Th2 memory cells, which has been suggested to be set during early life by a process of allergen driven Th1/Th2 selection, might be reflected by these see-sawing IgG subclass and IgE responses to different allergens. Prescott and colleagues, in a prospective two-year follow-up study, demonstrated a persistent proliferative response to house dust mite [13]. The cytokine responses of cord blood mononuclear cells (CBMC) showed a Th2-skewed profile in all children, but the Th2 responses were lower in individuals who later developed atopic diseases than in those who did not. Non-atopic individuals showed a gradual decline in detectable IL-4 mRNA and IL-13 protein responses to house dust mite, while atopic individuals had persistent IL-4 mRNA and IL-13 protein responses. There was a similar increase in house dust mite induced IL-6 and IL-10 production in the atopic and non-atopic groups. The IFN- γ mRNA responses were rarely detectable from CBMC but were easily detectable after six months of age in the non-atopic group but not in the atopic group.

So far, only allergen specific T-cell responses to house dust mite have been prospectively studied and then only up to two years of age [13]. The aim of this study was therefore to investigate the Th1/Th2 cell selection process to the seasonal inhalant allergen birch over several years. Birch is in some respect a model allergen, as the season of exposure is short, mostly less than six weeks.

Methods

Study group

Twenty-four families with their newborn babies were randomly invited at the maternity clinic at the University Hospital of Linköping, to participate in a prospective study regarding the development of immune responses to birch early in life. All children were born at term from July 1991 to March 1992. Twenty-one families accepted participation, and their children were followed annually during their first seven years of life. Clinical examinations, venous blood sampling and skin prick tests were performed during or shortly after each birch pollen season, i.e. between the middle of May and the middle of June, 1993-1998.

Atopic dermatitis was defined as pruritic, chronic, or chronically relapsing non-infectious dermatitis with typical features and distribution. Asthma was defined as four or more episodes of bronchial obstruction, at least once verified by a physician. Allergic rhinoconjunctivitis was defined as rhinitis and conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection. Skin prick tests were done in duplicate on the volar aspects of the forearms with thawed egg white, fresh skimmed cow's milk (lipid concentration 0.5%) and birch, timothy, cat, *Dermatophagoides*

pteronyssinus and *Dermatophagoides farinae* extracts (Soluprick®, ALK, Hørsholm, Denmark). Histamine hydrochloride (10 mg/ml) was used as a positive control, and albumin diluent was included as a negative control. The test was regarded as positive if the mean diameter was >3 mm.

The atopic status of the parents was established by a typical clinical history (*i.e.* allergic rhinoconjunctivitis, allergic asthma, or flexural itchy dermatitis).

Separation of PBMC

Blood samples were collected in tubes with preservative-free heparin (Beckton Dickinson, Stockholm, Sweden). Peripheral blood mononuclear cells were isolated on Ficoll Paque density gradient (Pharmacia Biotech, Sollentuna, Sweden), and washed three times in RPMI-1640 (Life Technologies AB, Täby, Sweden) containing 2% foetal calf serum (Life Technologies AB). They were then cryopreserved by standard methodology in 10% DMSO (Sigma-Aldrich, Stockholm, Sweden), 50% foetal calf serum and 40% RPMI-1640.

Proliferation

After thawing and washing, triplicate cultures of 2×10^5 PBMC in 200 μ l of AIM-V serum-free medium (Life Technologies) supplemented with 20 μ M mercaptoethanol (Sigma-Aldrich) were cultured at 37°C with 5% CO₂ with

medium alone or 10 000 Standard Quality Units (SQU)/mL Aquagen birch extract (ALK). Phytohemagglutinin (PHA) (Sigma-Aldrich), 5 μ g/mL, served as positive control. After five days, the cells were pulsed with 3 H-thymidine (Amersham, Stockholm, Sweden), and harvested 24 h later for detection of 3 H-DNA synthesis by liquid scintillation counting. Proliferative responses were expressed as a proliferative index i.e. counts per minute (cpm), divided by background responses (cultures with medium only). A proliferative index >2 was regarded as positive.

Cytokine production

After thawing, the cells were resuspended at 1×10^6 viable cells/mL AIM-V serum-free medium (Life Technologies) supplemented with 20 μ M mercaptoethanol (Sigma-Aldrich). One mL aliquots (1×10^6 cells) were cultured at 37°C with 5% CO₂ with medium alone, 10 000 SQU/mL Aquagen birch extract (ALK) or 2 μ g/mL PHA (Sigma-Aldrich). To enable measurement of IL-4, separate cultures were performed with monoclonal antibodies to human IL-4 receptor, 2 μ g/mL, (clone 25463.111, R&D Systems, Abingdon, UK). After 96 h, the samples were centrifuged at 2000 g for 5 min, the supernatants were aspirated and stored at -20°C. The cells were lysed with 300 μ l RLT Lysis Buffer (Qiagen, Hilden, Germany) and stored at -70°C.

ELISA for detection of IL-4, IL-5, IL-10, IL-13 and IFN- γ

The levels of IL-4, IL-10, IL-13 and IFN- γ were determined by commercially available ELISA kits (CLB Pelikine Compact™, Research Diagnostics Inc., Flanders, NJ), as described by the manufacturer. The levels of IL-5 were determined using an in-house ELISA as described elsewhere [14]. Briefly, Costar 3690 plates (Life Technologies AB) were coated with 50 μ L/well of 0.25 μ g/mL monoclonal rat anti-human IL-5 (clone TRFK5, Pharmingen, Becton-Dickinson). Free plastic spaces were blocked with 100 μ L/well of a blocking solution (CLB). Recombinant human IL-5 (Pharmingen, Becton-Dickinson) diluted two-fold (range 3.1-200 pg/mL) in AIM-V medium (Life Technologies) was used as a standard. Standards, samples, or for controls, AIM-V medium (Life Technologies) only, 50 μ L/well, were added to the plates. For detection, 50 μ L/well of a 1.0 μ g/mL monoclonal biotinylated rat anti-human IL-5 antibody was used followed by 50 μ L/well of streptavidin conjugated polyhorseradish peroxidase (CLB) diluted 1/10 000 in dilution buffer (CLB). Tetramethylbenzidine (Sigma-Aldrich), 50 μ L/well, was used as substrate and addition of 50 μ L/well of 1.8M sulphuric acid stopped the reaction. The sensitivity limit for quantitative determinations were 6.25 pg/mL for IL-4 and IL-5, 4.7 pg/ml for IL-10, 62.5 pg/mL for IL-13, and 25 pg/mL for IFN- γ .

RNA extraction and Reverse Transcription PCR of mRNA

Total ribonucleic acid (RNA) was isolated according to the RNeasy® 96 Protocol (Qiagen). In brief, the lysed cells were mixed with ethanol and applied to a RNeasy® 96 well plate. When the membrane had dried, the RNA was eluted in RNase-free water.

RNA was converted to complimentary DNA using an oligo dT-primer, random decamer primer and M-MLV reverse transcriptase for 1 h at 42°C followed by 10 minutes at 75°C according to the Reverse-it protocol of the supplier (Abgene Advanced Biotechnologies Ltd., Surrey, UK). The samples were run in a GeneAmp PCR System 2400 (Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Quantification of IL-4 and IL-9 mRNA with quantitative real time PCR

To a 96-well reaction plate (MicroAmp Optical, Perkin Elmer Applied Biosystems), 24 µl of Reaction Mix (TaqMan Universal PCR Master Mix, forward and reverse primers and probes, for concentrations see **table 1**) was added to each well followed by 1 µl samples, standards (cDNA from PHA stimulated mononuclear cells) or water as negative control. Oligonucleotide primers (MedProbe, Oslo, Norway) were designed for human IL-4 and IL-9 sequence using Primer Express (Perkin Elmer) (for sequences see table). The

TaqMan probe with the fluorophore FAM (6-carboxy-fluorescein) at the 5'-end and the quencher TAMRA (6-carboxy-tetramethylrhodamine) at the 3'-end were bought from MedProbe. Both primers and probe were purified with HPLC.

The wells were covered with optical caps (Perkin Elmer Applied Biosystems), and the plate was centrifuged for some seconds at high speed and thereafter placed into the thermocycler (ABI Prism™ 7700 Sequence Detector, Perkin Elmer Applied Biosystems). The thermal cycle conditions were 50°C for 2 minutes followed by 95°C for 10 minutes and then 40 cycles were run for 15 seconds at 95°C and for 1 minute at 60°C, as recommended by Perkin Elmer for the SDS 7700 system.

Samples were run with primers and probes for IL-4 or IL-9 and for rRNA in two different wells. The results from the rRNA reactions were used as internal controls, i.e. the amount of IL-4 and IL-9 mRNA was calculated relative to the amount of rRNA present in each sample.

Antibody analyses

Birch specific IgE antibodies were analysed with the high sensitivity protocol of Magic Lite™ according to the recommendations of the manufacturer (ALK).

The determination of IgG subclass antibodies to the recombinant major birch allergen Bet v 1 was performed as described in detail elsewhere [15]. Briefly, microtitre plates were coated with 1 μ g/ml rBet v 1 (kindly provided by Prof D Kraft, Institute of General and Experimental Pathology, AKH, University of Vienna, Austria) in PBS. Human serum or buffer only was added to duplicate wells followed by monoclonal murine antibodies to human IgG₁ (clone HP6069) and IgG₄ (clone HP6011), (Bio Zac AB, Järfälla, Sweden). Alkaline phosphatase-conjugated rabbit antimouse IgG (Sigma-Aldrich, Stockholm, Sweden) was then added, and p-nitrophenyl phosphate (Sigma-Aldrich) was used as substrate. The reference serum, which was calibrated according to a semiquantitative method as described earlier [15], was from a patient undergoing birch immunotherapy. Values were expressed as arbitrary units (AU) deduced from the ODs of the reference serum curve, after subtracting the blanks.

Samples with undetectable levels of IgG₄ antibodies with the conventional ELISA, (described above), were analysed with a more sensitive ELISA [15]. For the substrate reaction an amplified enzyme system, AMPAK (Dakopatts AB, Älvsjö, Sweden), was used.

Pollen counts

Total levels of birch pollen for each year were obtained from the Palynological Laboratory, Swedish Museum of Natural History. Birch pollen was counted in Norrköping, a city 40 km north of Linköping.

Statistics

As the concentrations of cytokines and antibodies were not normally distributed comparisons between paired groups were analysed using Wilcoxon Signed rank test and trends over time were analysed using ANOVA for repeated measurements. A probability level of <5% was considered to be statistically significant. Calculations were performed with a statistical package StatView 5.0 for PC (Abacus Concepts Inc., Berkely, California, USA).

Ethics

The Regional Ethics Committee for Human Research at the Linköping University approved the study.

Results

Sixteen of the children had at least one parent with a history of atopic symptoms (table 2). Eight children developed allergic symptoms during the six-year follow-up period, and are referred to as atopic. Seven of them were SPT positive (table 2). Two children developed symptoms of allergic rhinoconjunctivitis during the birch pollen season and persistent positive skin prick tests to birch pollen extract from age four (table 2). They were the only children who had measurable IgE to birch (fig 1). These two children are referred to as birch allergic.

Proliferative responses to birch were detected in most atopic and non-atopic children, already during their second pollen season in 1993, and the magnitude of the responses was similar over the following years (fig 2).

Similarly, birch induced IFN- γ production was detected in most of the children during the second pollen season (fig 3a). The levels were largely similar up to age five and then increased. The birch induced IFN- γ levels increased statistically significantly from 1993 to 1997 and 1998 ($p=0.01$ and 0.03 , respectively, Wilcoxon Signed Rank test). Birch induced IL-10 production increased during the first years of life (fig 3b). The IL-5 and IL-13 production

(fig 3c-d), on the other hand, tended to decrease with age. This was statistically significant from 1995 to 1998 ($p=0.02$) and from 1996 to 1998 ($p=0.03$) for IL-5 and from 1993 to 1995, 1996 and 1998 ($p=0.02$, 0.04 and 0.048, respectively, Wilcoxon Signed Rank test) for IL-13. Two children, however, showed persistent birch induced IL-5 responses (fig 3c). Both of them developed allergic symptoms and had positive SPT to birch from age four. One of the two birch allergic children also showed persistent birch induced IL-13 production (fig 3d). Birch induced IL-4 protein production and mRNA expression, as well as IL-9 mRNA expression, was only demonstrated in some children. The age related decrease of IL-9 mRNA expression reached statistical significance from 1994 to 1997 and 1998 ($p=0.049$ and 0.046, respectively), from 1995 to 1997 and 1998 ($p=0.004$ and 0.01, respectively) and from 1997 to 1998 ($p=0.02$, Wilcoxon Signed Rank test). Only the two birch allergic children showed persistent IL-4 protein production (fig 3e), IL-4 mRNA (data not shown) and IL-9 mRNA (fig 3f) expression.

IgG₁ antibodies to rBet v1 were commonly detected both in atopic and non-atopic infants from the second pollen season and the levels increased with age. The four children with the highest levels of IgG₁ antibodies all had allergic symptoms and two of them were allergic to birch (fig 4a). In contrast, IgG₄

antibodies to rBet v1 were only sporadically demonstrated, and only the two birch allergic children showed high and persistent IgG4 levels (fig 4b).

The levels of birch specific IgE antibodies in the two birch allergic children, seemed to be related to the counts of birch pollen from the third pollen season in 1994 (fig 1). There was no association between pollen counts and birch induced proliferation or cytokine production or the levels of IgG antibodies to birch, however (fig 2-4).

Discussion

In this prospective study we show that immune responses to birch, during the first seven years of life, are both Th1- and Th2-like. Thus, birch induced IFN- γ (Th1) and IL-4, IL-5 and IL-13 protein and IL-4 and IL-9 mRNA (Th2), as well as Th2 associated IgG₄ production was documented, both in atopic and non-atopic children. The two children who developed allergy to birch were the only to have persistent IL-4, IL-5, IL-9, IgE and IgG₄ responses to birch, indicating a failure in down-regulating the neonatal Th2-like immunity. Thus, our study confirms and extends previous studies showing that postnatal Th2-like responses to allergens are common, independent of the atopic status later in life, and that these responses are later down-regulated, except in children who develop clinical symptoms to the allergen [10, 11, 16].

The failure of immune deviation to allergens among atopic individuals may be associated with poor neonatal capacity to produce IFN- γ [17, 18]. The IFN- γ producing capacity is much lower in neonatal T cells than in adults, and production increases with age [19]. The low IFN- γ production during infancy might be due to postnatal persistence of control mechanisms limiting intrauterine induction of potentially harmful Th1-like immunity [20]. In this prospective study, we show a similar birch induced IFN- γ production in atopic

and non-atopic children. This is in agreement with a recent prospective study where house dust mite induced IFN- γ production was low both in atopic and non-atopic children during their first 18 months of life [13]. In that study, however, house dust mite induced IFN- γ mRNA expression increased with age only in the non-atopic individuals.

Although allergen specific Th1-like immunity dominates in healthy individuals, IFN- γ associated delayed type hypersensitivity is not observed. Factors counteracting Th1 reactions are likely to be present and the anti-inflammatory cytokine IL-10 may be one candidate [9]. House dust mite specific IL-10 production from CBMC is upregulated in non-atopic, as compared to atopic, individuals [13]. The age dependent increase of IL-10 production was similar, however, in the two groups. Our results are consistent with that finding, as IL-10 production was detected in all children from the fourth pollen season.

The IL-4, IL-5 and IL-9 responses to birch declined with age, except in the two birch allergic children. The allergen induced production of those cytokines has not previously been prospectively studied at the protein level. Similarly, IL-13 responses declined with age except in one of the two birch allergic children. Although IL-13 induces IgE switching independently of IL-4 [21], it is not a typical Th2 cytokine, as both Th1 and Th2 clones produce it and it is not capable

of inducing Th2 differentiation. No clear difference in IL-13 production was observed between atopic and non-atopic children in this study. In contrast, house dust mite induced IL-13 production is down-regulated in non-atopic children, whereas the opposite was observed for atopic children, independently of what allergen the child was sensitised against [13]. There are, however, also differences in the methodology. In the study by Prescott *et al*, the cells were incubated with allergen for 24 h, in contrast to 96 h in our study. It has earlier been shown that allergen induced IL-13, as well as IL-5 and IFN- γ production increase over time and that the levels and number of individuals with detectable levels were significantly higher after 96 h, as compared to 24 h, of incubation [12]. Furthermore, the atopic status of the children in the study by Prescott *et al* was based on a two-year follow-up whereas the children in our study were followed during their first seven years of life.

The IgG responses to Bet v 1 were largely restricted to the IgG₁ subclass, confirming the findings in a previous cohort [15]. The IgG₁ responses were commonly seen, regardless of atopic symptoms and sensitisation but, as reported previously, they were higher in atopic children [15, 22]. Similar to findings in another cohort [11], there was an even stronger relationship between Th2 associated IgG₄ antibodies and atopy. In that study, the differences in IgG₄ antibody levels between atopic and non-atopic children were greater at 8 years

than at 18 months, which was largely due to down-regulation of IgG4 antibody responses in non-atopic children. This is supported by the findings in the present study.

The period when allergen driven Th1/Th2 selection occurs, the so-called “window of sensitisation” has been suggested to occur during a narrow period after birth [23]. We have with this study extended this hypothesis to suggest that this may last for around 3-4 years after birth, at least for a seasonal allergen with a short period of exposure every year.

In conclusion, immune responses to birch can be demonstrated in children regardless of atopic status. A transient early Th2-like response is down-regulated after the fourth pollen season, except in children who develop clinical allergy to the particular allergen.

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Legends to figures

Fig 1

IgE antibodies to birch in sera and birch pollen counts (grey bars) over the first seven years of life were only detected in the two children who developed allergy to birch. The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. Blood samples were collected during each pollen seasons of 1993-1998. The number of analysed samples is indicated (n).

Fig 2

Birch induced proliferation from peripheral blood mononuclear cells and birch pollen counts (grey bars) over the first seven years of life in 8 atopic (open circles) and 13 non-atopic children (filled circles). The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. The children with positive SPT to birch in each year are indicated with *. Blood samples were collected during each pollen seasons of 1993-1998. The number of analysed samples is indicated (n), as well as the median.

Fig 3

Birch induced IFN- γ (a), IL-10 (b), IL-5 (c), IL-13 (d), IL-4 (e) and IL-9 (f) responses from peripheral blood mononuclear cells and birch pollen counts (grey bars) over the first seven years of life in 8 atopic (open circles) and 13 non-atopic children (filled circles). The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. The children with positive SPT to birch in each year are indicated with *. Blood samples were collected during each pollen seasons of 1993-1998. The p value for trends were <0.05 for IL-10 (b). The number of analysed samples is indicated (n), as well as the median.

Fig 4

Titers of IgG1 (a) and IgG4 (b) antibodies to rBet v 1 in sera and birch pollen counts (grey bars) over the first seven years of life in 8 atopic (open circles) and 13 non-atopic children (filled circles). The values of the two children who developed allergy to birch are connected with dotted lines and are indicated with A and B. The children with positive SPT to birch in each year are indicated with *. Blood samples were collected during each pollen seasons of 1993-1998. The p value for trends were <0.05 for IgG1 (a). The number of analysed samples is indicated (n), as well as the median.

Table 1. Sequences of primers and probes

Target	Primer/ probe	Sequence (5' to 3')	Conc (nM)	Positio n	Amplico n length
rRNA	FP	CGGCTACCACATCCAAGGAA	200	*1	*1
	Probe	TGCTGGCACCAGACTTGCCCTC	50	*1	*1
	RP	GCTGGAATTACCGCGGCT	100	*1	*1
IL-4	FP	ACAGCCTCACAGAGCAGAAG ACT *2	300	178-201	72
	Probe	TGTGCACCGAGTTGACCGTAACAGAC	200	203-229	
	RP	A TGT TCTT GGAGGCAGCAAAGA	300	250-229	
IL-9	FP		300	128-148	76
	Probe	GATCCAGCTTCCAAGTGCCA AAACAGAGACA ACT GGTCACATTAGC	100/ 175		
	RP	ACTGCA TGCAGTTGTCAGAGGGGAATGC		204-181	

FP = Forward primer

RP = Reverse primer

*1 = Recommended by PE Biosystems

Letters in bold indicate an exon-exon junction

Table 2. Data regarding development of atopic dermatitis (AD), allergic rhinoconjunctivitis (ARC) and asthma (AB) and skin prick test (SPT) positivity in the individual babies and the symptoms reported in the mother (M) and father (F).

Patient	Symptoms	SPT positivity	Atopic heredity
1			F (AD)
2		Egg -93, -94	M (ARC)
3	AD	Egg -93-97	M (ARC)
5			M (ARC)
6		Birch-94, -95	F (ARC)
7			
8	AD, ARC, AB	Egg -93-97, Birch, Cat, Timothy -95-98	M (ARC)
11		Timothy -96,-98	
12	AD	Milk, Cat -93, -95	M, F (ARC)
13			M (AD, ARC), F (ARC)
14	AD, AB		M (AD), F (ARC)
15	AD		M, F (ARC)
16	AD		M (AB)
17		Birch -98	M (ARC)
18			
19		Birch -94	M (ARC?)
20			
21		Birch -94	
22	AD	Birch -95	F (ARC)

23	ARC	Birch, Cat -95-98, Timothy -97-98	M (ARC)
24			M, F (ARC)

Fig 1

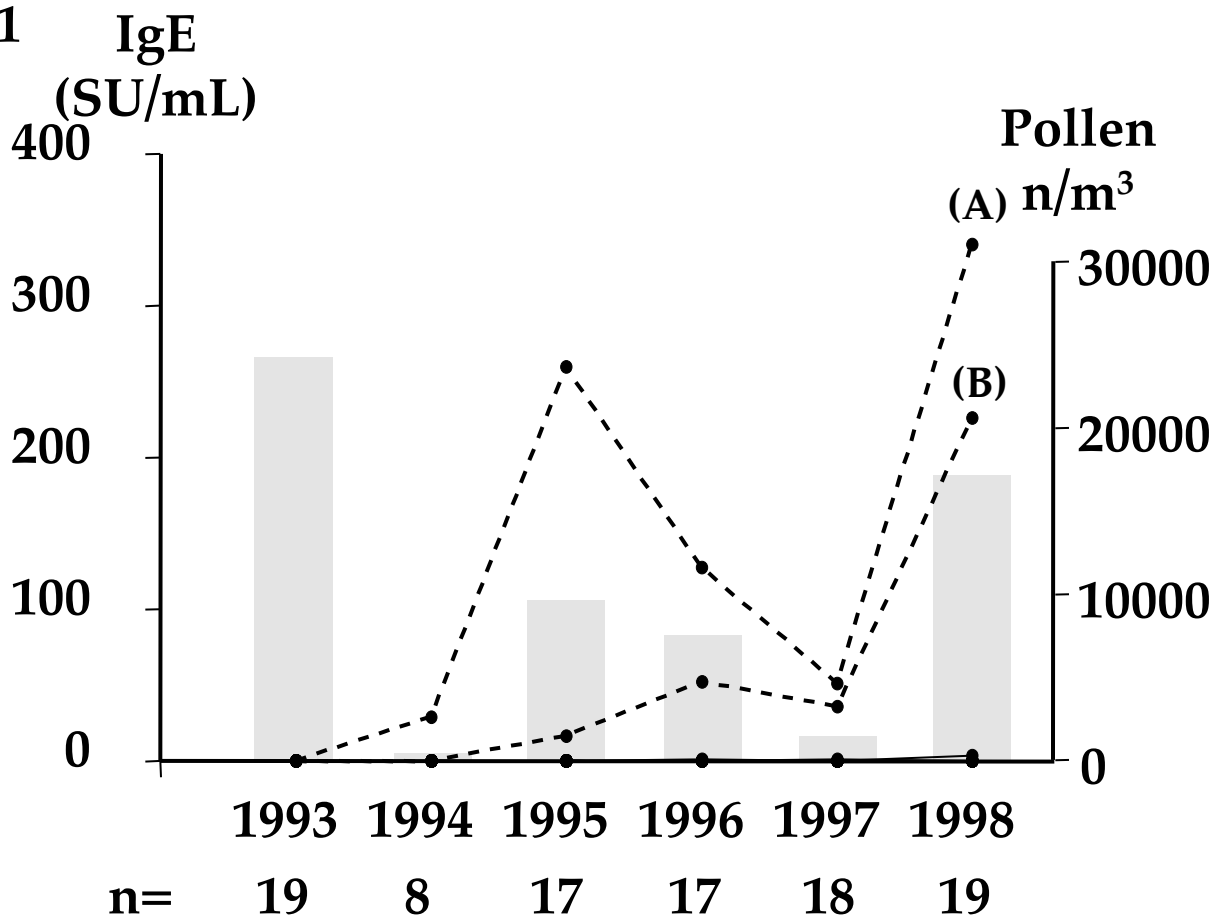


Fig 2

Proliferation index

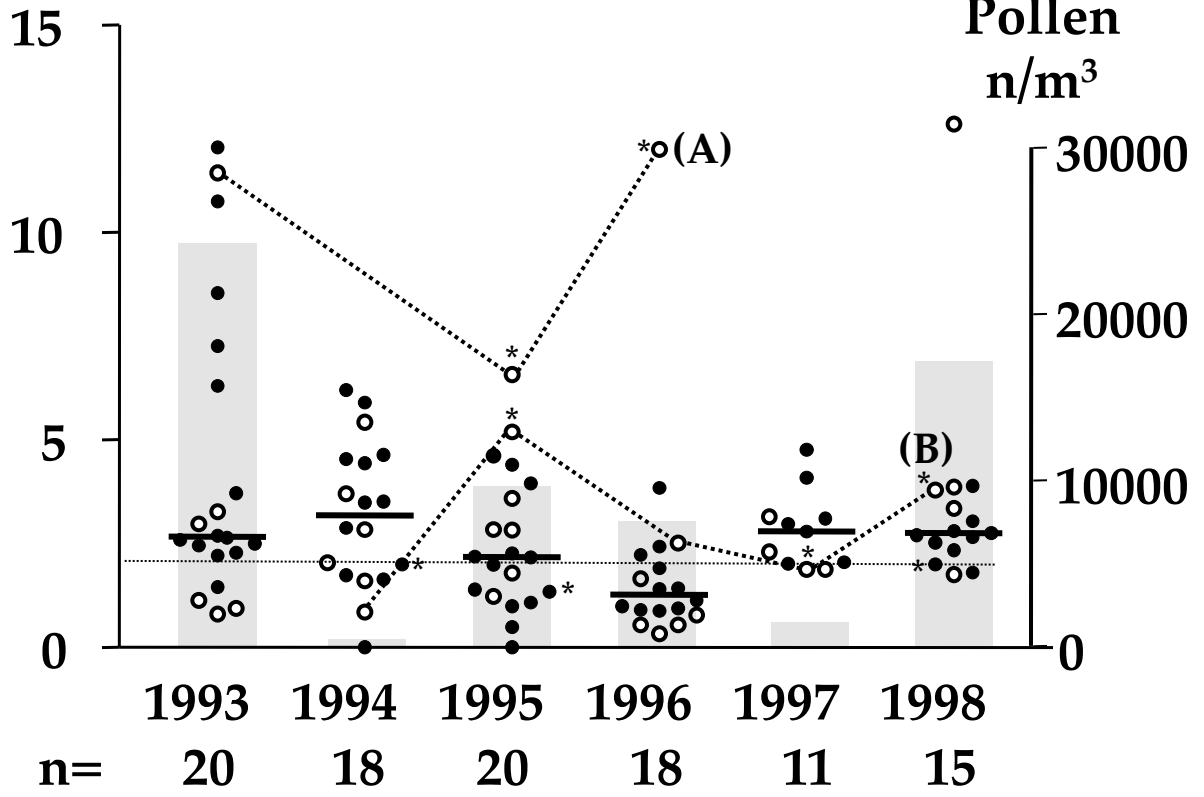


Fig 3a

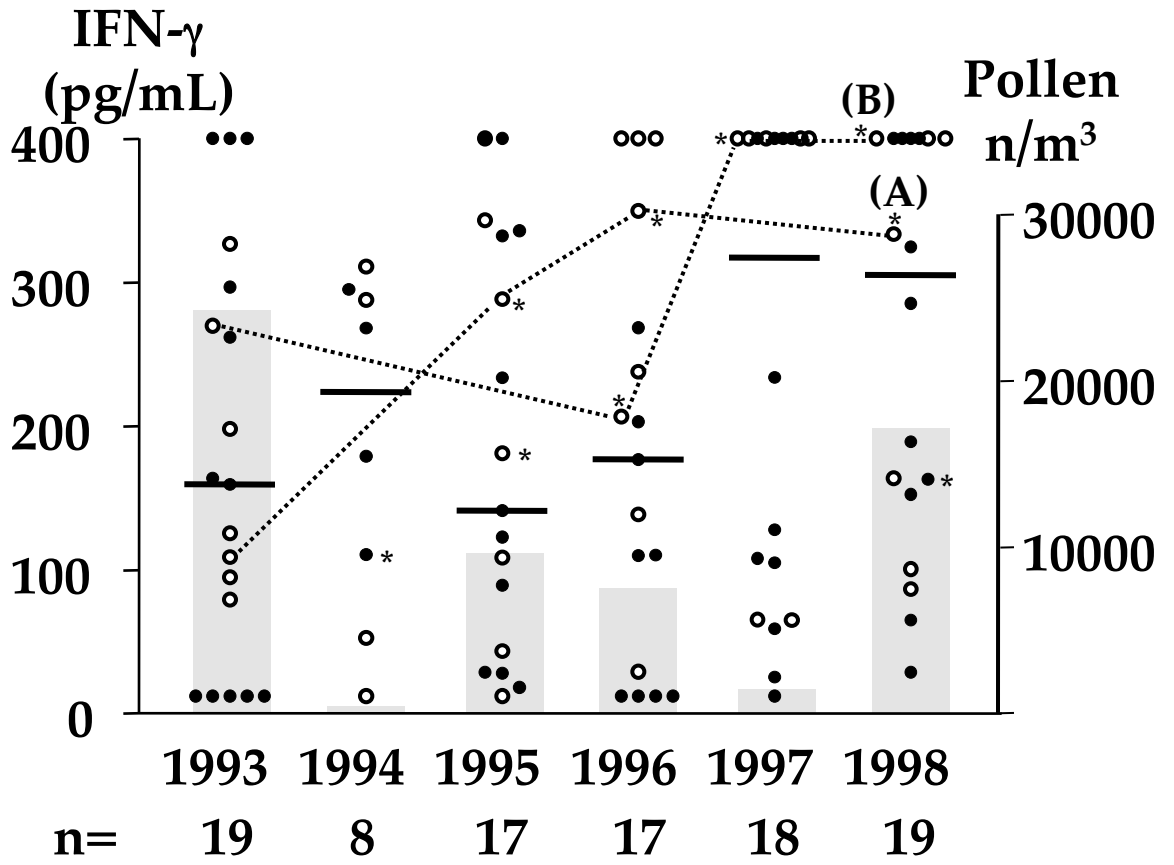


Fig 3b

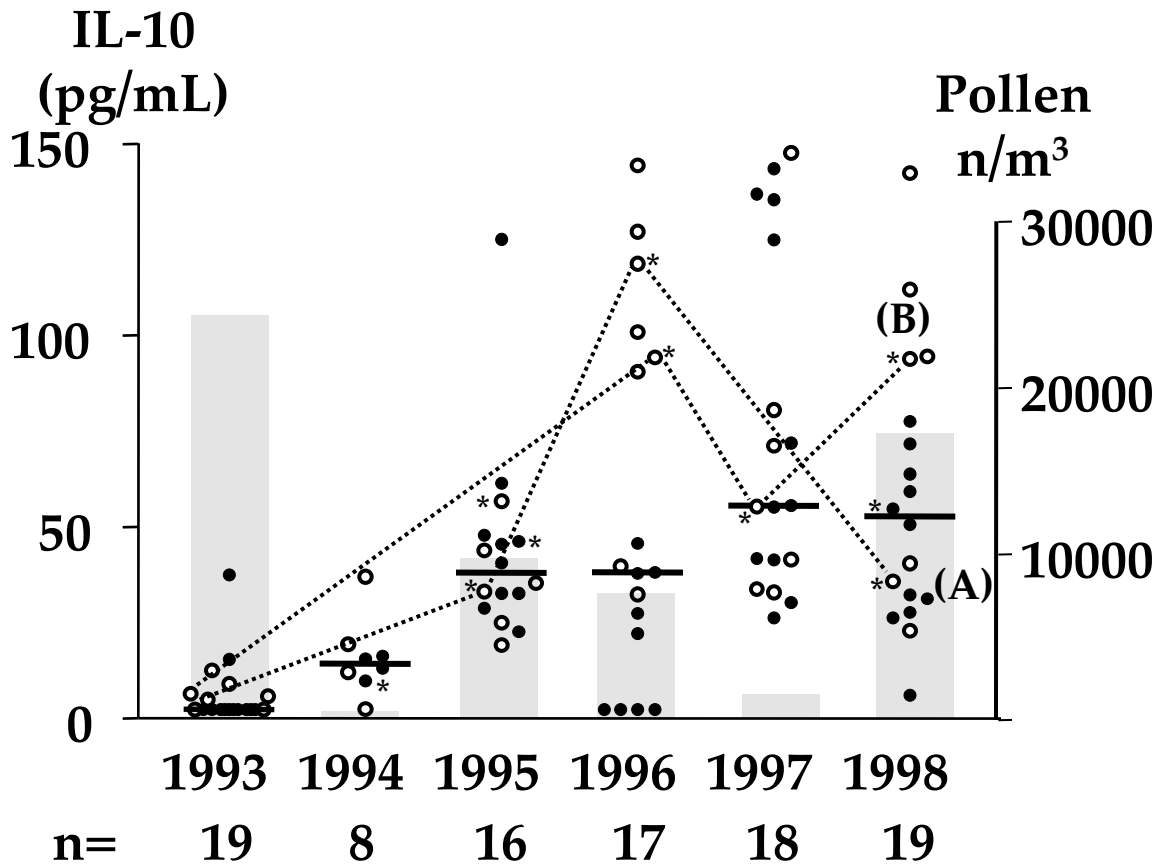


Fig 3c

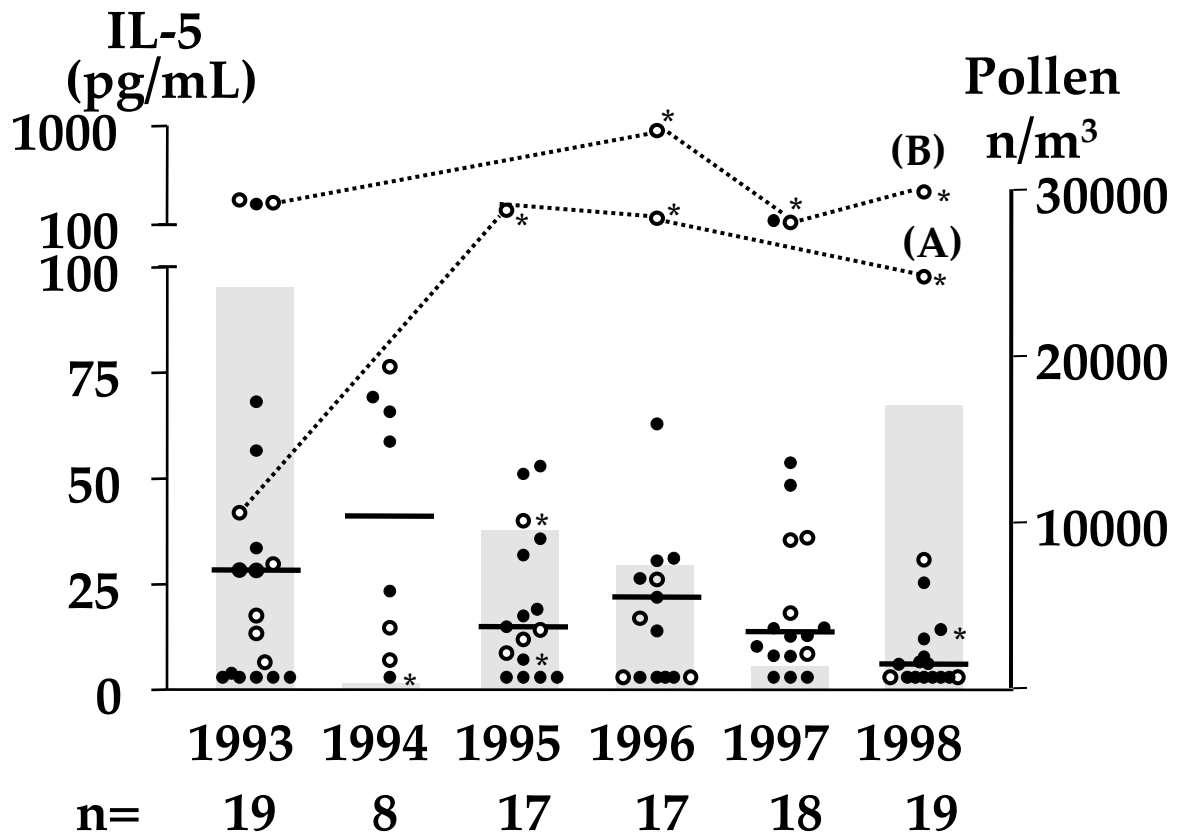
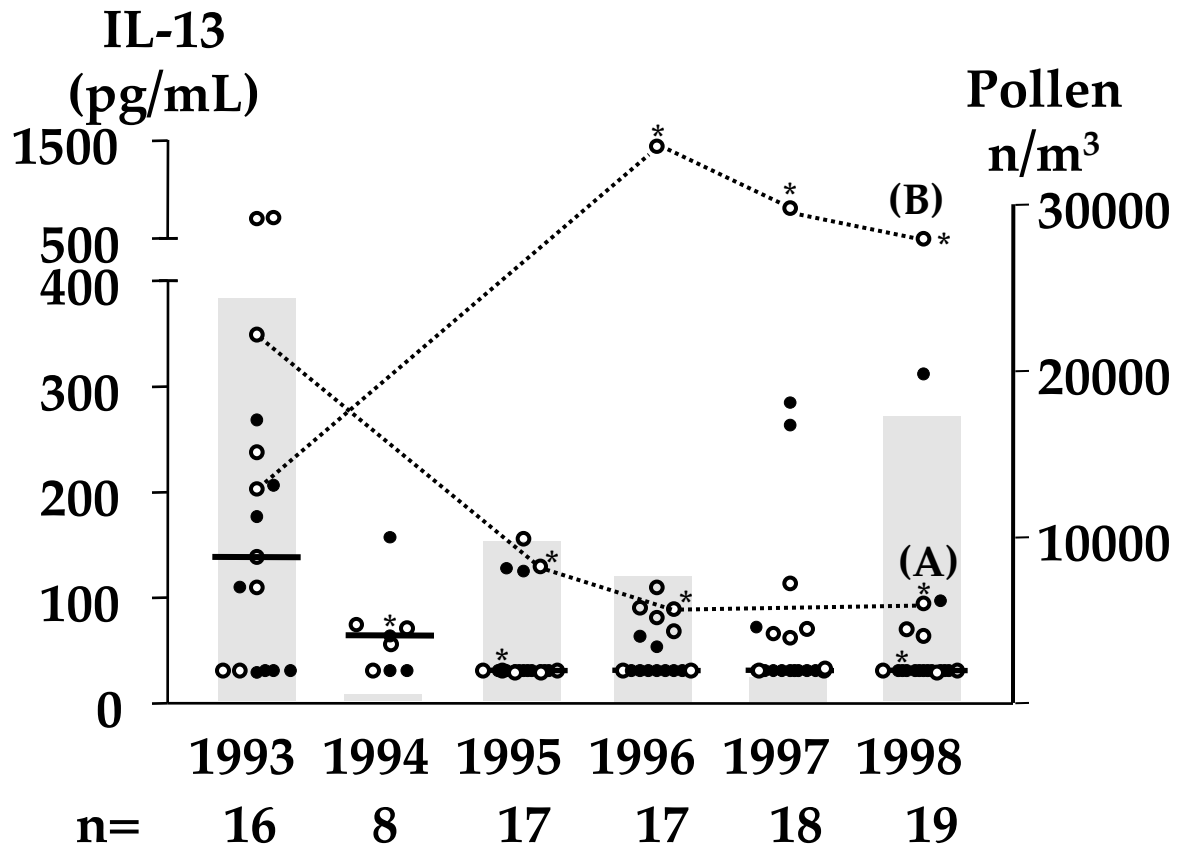


Fig 3d



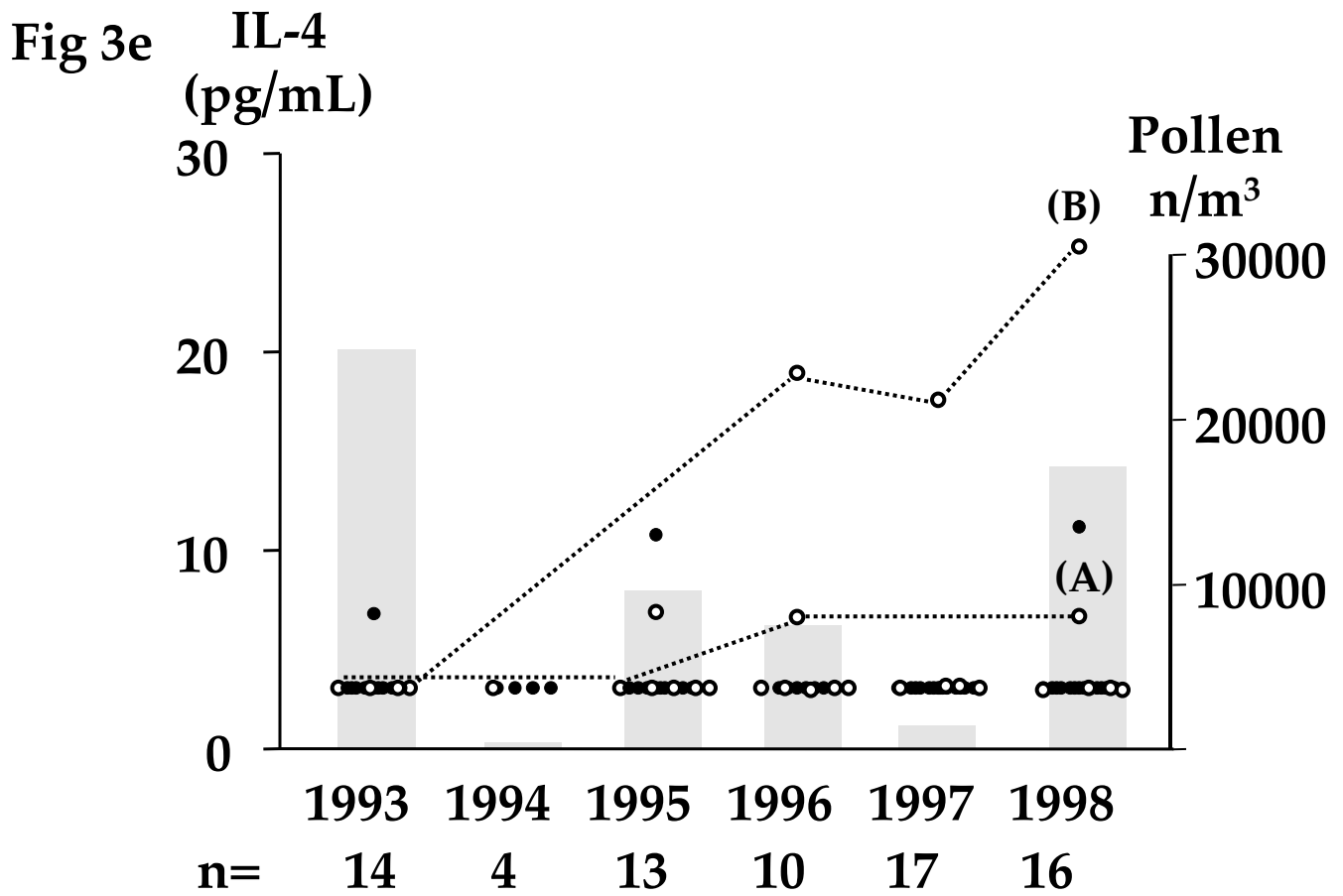


Fig 3f

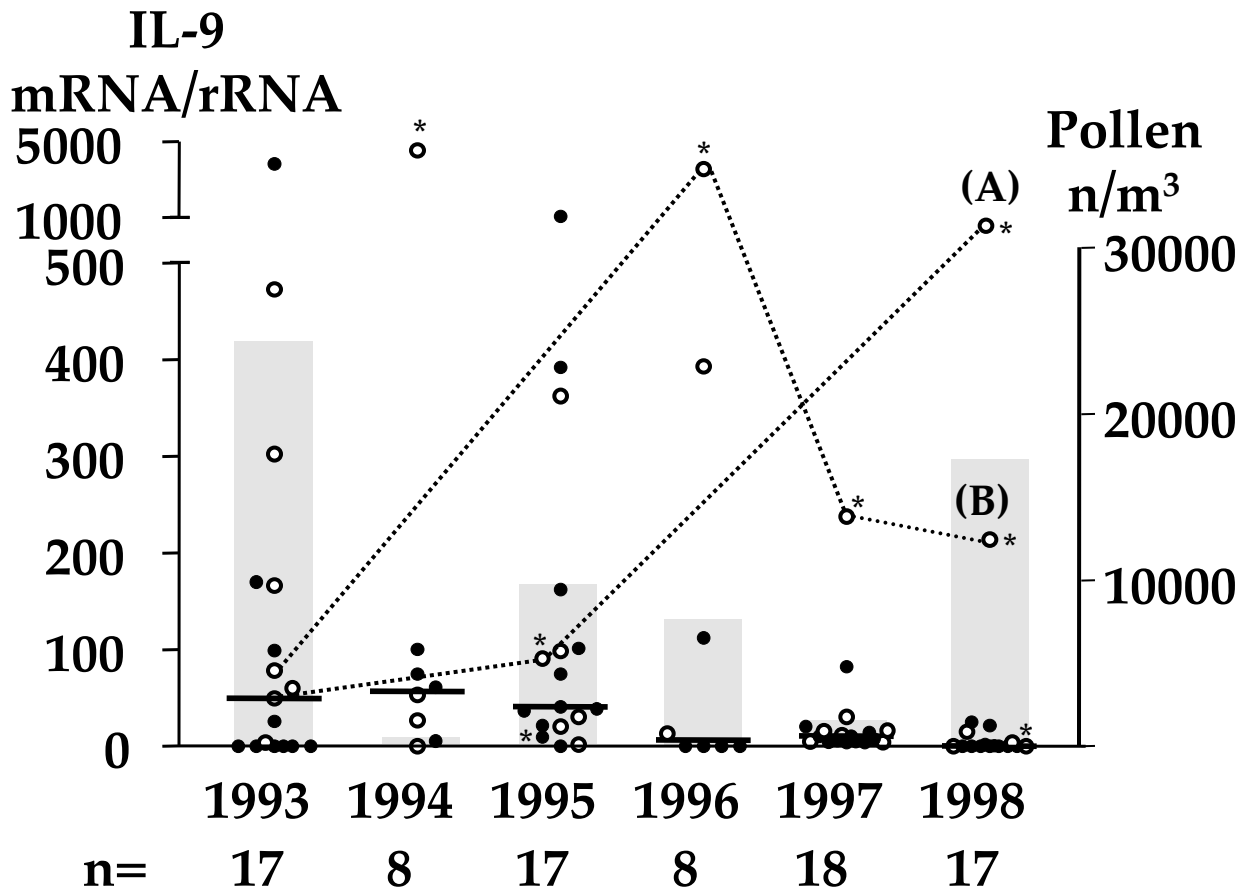


Fig 4a

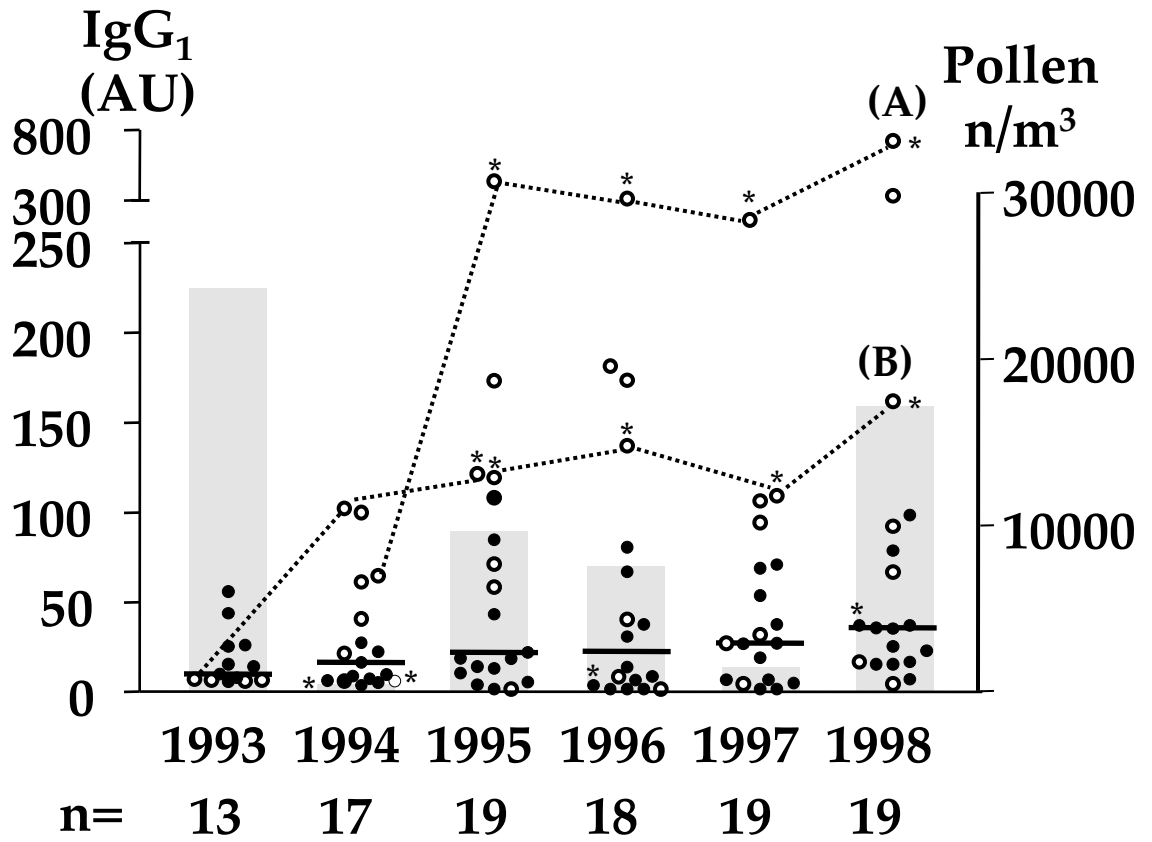


Fig 4b

