

Mouse Strain Specificity of the IgE Response to the Major Allergens of *Phleum pratense*

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Key Words

High responder · IgE · Low responder · Mouse model ·
Phleum pratense

Abstract

Background: IgE immune responses against major allergens from *Phleum pratense* in low- and high-responder mouse strains were compared and the influence of alum was assessed, in order to evaluate the effect of the genetic background and adjuvants on IgE reactivity in a mouse model for *P. pratense* allergy. **Methods:** Different mouse strains and F1 offspring were sensitized with *P. pratense* pollen extract. Serum IgE levels, the induction of specific IgE antibodies and immediate cutaneous hypersensitivity reactions were monitored by ELISA, Western blot and a skin test, respectively. **Results:** All mouse strains investigated mounted an IgE response and exhibited a positive skin test to pollen extract. Differences were seen in the level of total serum IgE and in specific IgE reactivity to different major allergens of *P. pratense*. Notable differences were seen in IgE reactivity and immediate hypersensitivity against Phl p 1, which were only observed in SJL/j mice. The foremost influence of alum was on total

IgE production levels. **Conclusions:** Alum is not necessary as an adjuvant to elicit IgE reactivity against the clinically relevant allergens of *P. pratense*, since even low-responder mouse strains mounted a hypersensitivity reaction after sensitization without the adjuvant using otherwise identical sensitization strategies. Moreover, when analyzing the allergenicity of a compound, the hypersensitivity response of different mouse strains should be considered, as implicated by the differential results obtained for IgE reactivity against Phl p 1. Lastly, a genetic component may be involved in IgE reactivity to Phl p 1.

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Introduction

Environmental and genetic factors influence the development of allergic disease. Type I allergic reactions to grass pollen have been described in up to 25% of the inhabitants of industrialized countries, with increasing tendency [1, 2]. In a European study on asthma, allergy, and allergic sensitization in adults (European Community Respiratory Health Survey), the prevalence of asthma reached up to

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1018–2438/05/1364–0347\$22.00/0

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11.9%, that of allergic rhinitis up to 40.9%, that of bronchial hyperreactivity up to 27.9% and the prevalence of allergic sensitization against common aeroallergens attained up to 44.5% in Germany [3]. Although the exposure to the seasonally ubiquitous aeroallergens is quite similar, only a small group of a given population develops immediate-type hypersensitivity after repeated contact with aerosolized grass pollen. Moreover, varying degrees of IgE reactivity to different allergens have been shown. With respect to the major allergens from *Phleum pratense*, the highest percentage of patients reacts with Phl p 1 (96% [4, 5]) followed by Phl p 5 (80% [4, 5]) and Phl p 6 (75% [6]).

Several mouse models have been established which allow studies regarding the induction of allergic phenotypes analogous to the human disease. These models have shown that different allergic features such as IgE production, allergen-specific induction of T cells, induction of immediate cutaneous hypersensitivity reaction (ICHR) and airway hyperreactivity can be elicited in the mouse. Furthermore, the results of these experiments pointed out that some mouse strains are high responders, while others are intermediate or even low responders with respect to IgE synthesis. Saloga et al. [7] demonstrated that ovalbumin-sensitized BALB/c but neither naive mice nor ovalbumin-sensitized SJL mice were able to mount an IgE response and to develop ICHR and thus characterized BALB/c as high responders and SJL mice as non-responders. In another study, Daser et al. [8] demonstrated that, based on their capacity to develop ICHR against birch pollen, mice with different genetic backgrounds can be identified as low (C57BL/6) or high (A/J) responders.

Recently, we have described the IgE response of BALB/c mice to *P. pratense* pollen extract (PE) [9] and demonstrated that the IgE response to *P. pratense* allergens strongly resembles the one found in human patients, differing only in the lack of an IgE reactivity against Phl p 1 [9, 10]. To our knowledge, there is no study dealing with grass pollen allergens in association with the induction of IgE and ICHR to these clinically relevant allergens in different mouse strains. As human atopic patients exhibit their disease on the background of genetic predisposition, allergen exposure and environmental factors, we investigated the IgE response to PE in mouse strains previously categorized as high and low responders [7] and in F1 progeny of these strains. The purpose was to assess the magnitude and the specificity of the IgE response with and without aluminum hydroxide gel [Al(OH)₃, alum] and to investigate the functional relevance with respect to ICHR. Investigations with F1 progeny were included to evaluate the influence of the genetic

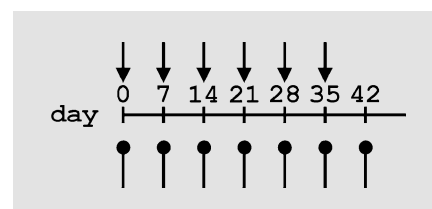


Fig. 1. Immunization scheme of mice with *P. pratense* pollen extract. Mice were treated every 7 days (arrows) and were bled correspondingly (circles).

background on these read-out parameters for type I allergy. The aim of the investigations was to resolve the suitability of different mouse strains for their use as models for pollen allergy.

Materials and Methods

Experimental Animals

Experimental animals were matched for age and sex. Female mice, SJL/j (SJL/JOrllcoCrIBR; H2 haplotype s), BALB/c (BALB/cAnNCrIBR; H2 haplotype d), C57BL/6 (C57BL/6NCrIBR; H2 haplotype b), B6SJLF1/J (C57BL/6 F × SJL/j M F1 hybrid) and CSJLF1/J (BALB/c F × SJL/j M F1 hybrid), were purchased from Charles River Laboratories (Sulzfeld, Germany). According to the supplier, the animals were certified as pathogen and virus free, were kept in a standard environment and fed with a maintenance diet and water ad libitum. Experiments were performed according to the national animal protection guidelines and were approved by the governmental authority (Animal Protection Authority of the Ministry of Environment, Kiel, Germany).

Preparation of *P. pratense* Pollen Extract and Purification of Phl p 1

P. pratense pollen was purchased from ARTU Biochemicals (Leystad, The Netherlands). The preparation of the pollen extract was performed as previously described [11]. Briefly, pollen was solubilized in 25 mM NH₄CO₃ (pH 8), stirred for 30 min at room temperature and after a centrifugation step followed by crude filtration the supernatant was excessively dialyzed against water. The dialysate was aliquoted, lyophilized and stored at –20°C until use. For injection or cell culture the lyophilisate was reconstituted with PBS (137 mM NaCl, 3.9 mM KH₂PO₄, 16 mM Na₂HPO₄ × 2 H₂O, pH 7.2) at 1 mg/ml and sterilized by filtration (pore size 0.2 µm).

For the purification of Phl p 1, a previously described procedure was used [12], which efficiently separates this allergen from other major allergens found in the extract.

Sensitization of Mice with *P. pratense* Pollen Extract

Animals were intraperitoneally injected with 100 µg PE with or without (w/o) 50 µg hydrated Al(OH)₃ gel (alum, Deutsche Tierärzte Genossenschaft, Serumwerke Memmsen, Memmsen, Germany) in PBS in a total volume of 200 µl. Adsorption was achieved during a

30-min incubation at room temperature on a rotator. Intraperitoneal injections were repeated every 7 days for up to 6 times (fig. 1). Control animals were either treated with alum alone or with PBS. Tail vein blood was collected for serum preparation and was kept at -20°C until use.

Immunoblotting for the Detection of Specific IgE Antibodies

Proteins were separated by 12.5% SDS-PAGE and were blotted onto nitrocellulose membranes (pore size $0.2\ \mu\text{m}$, Roth, Karlsruhe, Germany). Nitrocellulose membranes were cut into strips and incubated with the respective murine sera. Antigen-bound serum IgE was detected with a rat anti-mouse IgE monoclonal antibody (R35-72, BD Biosciences-Pharmingen, Heidelberg, Germany) and developed using a secondary goat anti-rat antibody coupled to alkaline phosphatase (Dianova, Hamburg, Germany). NBT/BCIP was used as the chromogenic substrate. Strips incubated with the secondary antibodies alone served as negative controls. The major allergens which elicit an IgE response in BALB/c mice have been determined previously [9].

Detection of Total Serum IgE and Specific Immunoglobulin Subclasses by ELISA

For detection of total serum IgE, standard microtiter plates (NUNC Maxisorp F 96, No. 44 2404, Nunc, Wiesbaden, Germany) were coated with a rat-anti murine IgE monoclonal antibody (clone R35-72, BD-Biosciences Pharmingen). Murine serum (1:15 dilution) was added, and IgE antibodies were detected after binding to the capture antibody with a biotin-conjugated rat anti-mouse monoclonal antibody (clone R35-92, BD-Biosciences Pharmingen). Bound antibody was visualized using streptavidin-coupled alkaline phosphatase (No. 016-050-084, Dianova) and *p*-nitrophenylphosphate (Sigma, Deisenhofen, Germany) as the substrate. Extinction at 405 nm was measured in an ELISA reader. Clonal murine IgE (clone IgE-3, BD-Biosciences Pharmingen) was used as a standard for quantification. The level of detection was 5–10 ng/ml.

Pollen extract-specific immunoglobulin levels were determined using pollen extract-coated plates and the respective biotinylated or alkaline-phosphatase-coupled specific anti-mouse immunoglobulin reagent (rat anti-mouse IgG1, clone A85-1; rat anti-mouse IgG2a, clone R19-15; rat anti-mouse IgG2b, clone R12-13, all from BD-Biosciences Pharmingen). Sera were added in serial dilutions from 1:50 to 1:36,000. Values shown in the results are from 1:450 dilutions for IgG2a and IgG2b and from 1:12,000 dilutions for IgG1.

Detection of Immediate Cutaneous Hypersensitivity Reactions

Detection of ICHR was performed as previously described [10]. Briefly, 1 week before skin testing, the animals were challenged by intranasal application of $5\ \mu\text{g}$ pollen extract or $1\ \mu\text{g}$ purified Phl p 1. In order to visualize the effects of mast cell degranulation and consecutive wheal-and-flare reactions of the skin, the fur of the abdomen was removed the day before the test using a depilatory cream (WDT, Garbsen, Germany). Fifteen minutes before the test, animals received intravenous injections of $100\ \mu\text{l}$ of a 0.5% Evans blue/PBS solution and were anesthetized by intramuscular injection of $25\ \mu\text{l}$ of a mixture of ketamine ($45\ \text{mg/kg}$ body weight) and Rompun[®] ($8\ \text{mg}$ xylazine hydrochloride/kg body weight). The allergens to be tested were dissolved in 0.9% NaCl and injected intradermally in a total volume of $20\ \mu\text{l}$ at doses of 5 and 75 ng. The histamine-releasing factor compound 48/80 (Sigma) served as a positive and 0.9% NaCl as a negative control.

The blue staining reactions were read and photographed 15 min after the injections. Blue staining reactions were rated as positive or negative in comparison to the respective controls.

Statistical Analysis

Wilcoxon's matched pair test or one-way analysis of variance (ANOVA) was used to determine statistically significant differences between groups. For the latter, comparisons of all pairs were performed using Tukey honestly significantly different (HSD) and Bonferroni tests, and Tukey HSD was used to test the homogeneity of subsets used in the analyses. *p* values <0.05 were considered statistically significant.

Results

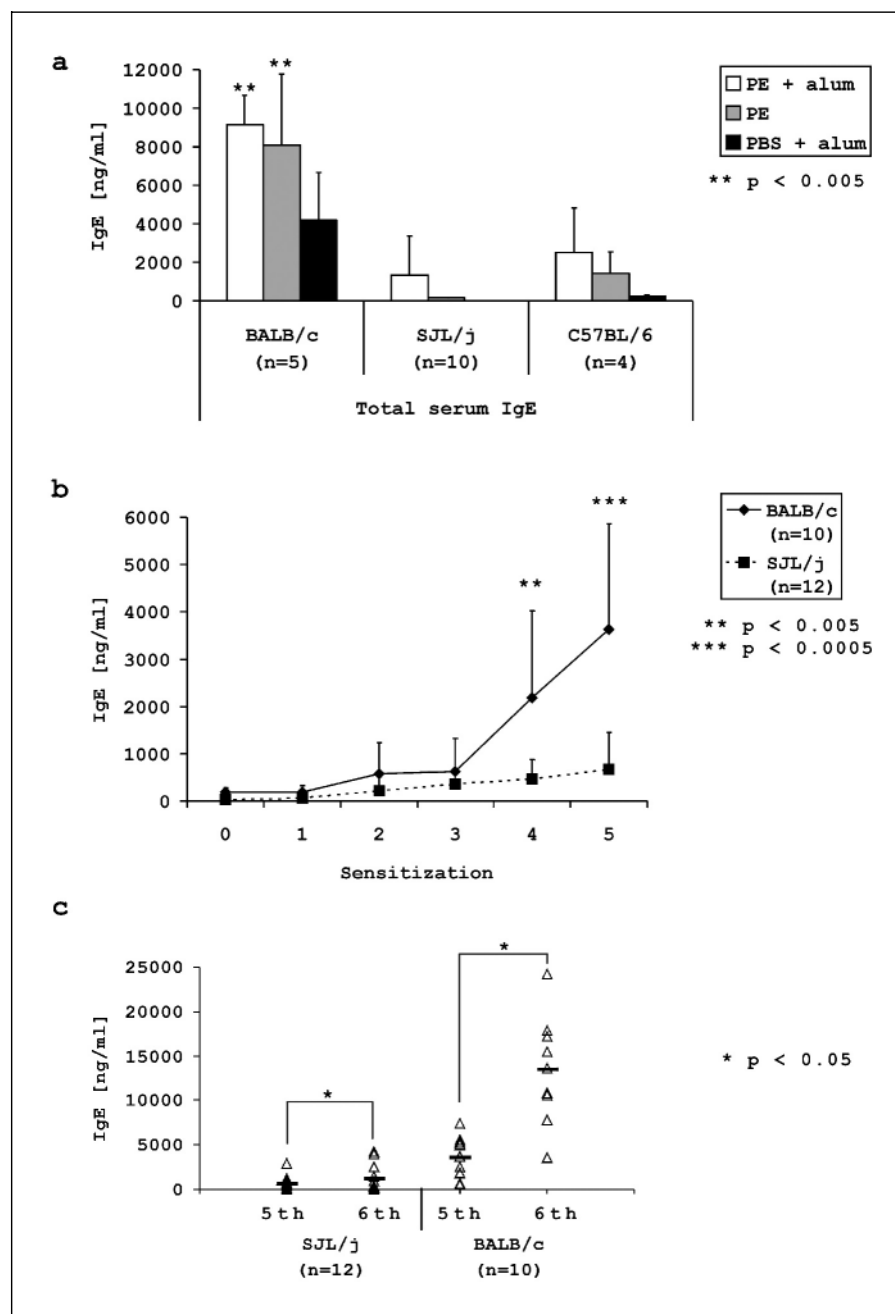
Total Serum IgE Levels in Mice Sensitized with Pollen Extract and $\text{Al}(\text{OH})_3$

All three mouse strains investigated showed an increase in total serum IgE levels after the 6th sensitization with PE and alum but also with PE without alum. BALB/c mice mounted the highest total IgE response, whereas C57BL/6 showed intermediate and SJL/j low levels of IgE (fig. 2a). The IgE levels found in BALB/c mice were significantly higher than in the SJL/j and C57BL/6 mice ($p < 0.005$). This experiment also showed that alum alone had a noticeable effect on IgE levels, especially in BALB/c mice; the effect was decreased in C57BL/6 mice and not detectable in SJL/j mice. The investigation of the time course of IgE production showed that no significant differences were evident after three sensitizations between BALB/c and SJL/j mice (fig. 2b). After the 5th and 6th sensitization, IgE production in BALB/c mice increased significantly, reaching levels five times higher than in SJL/j mice. Interestingly, however, BALB/c mice, which are classified as high IgE responders [13], showed a high variation in IgE production, which was most marked after the 6th sensitization and not significantly different from the 6th sensitization in SJL/j mice (fig. 2c). Remarkably, 30% of the SJL/j mice, which are categorized as low IgE responders [14], proved to be comparatively moderate responders in terms of IgE production after the 6th sensitization, with some SJL/j mice producing even higher IgE levels in response to pollen extract sensitization than some BALB/c mice after the 5th sensitization (fig. 2c).

Specific IgE Reactivity to Major Allergens of Mice Sensitized with Pollen Extract and $\text{Al}(\text{OH})_3$

All strains investigated showed a specific IgE reactivity against the major allergens Phl p 5a, 5b, 4 and 13. However, only SJL/j mice demonstrated IgE reactivity to Phl p 1 (fig. 3a). The IgE reactivity against Phl p 1 was verified

Fig. 2. Total serum IgE levels of different mouse strains sensitized with *P. pratense* pollen extract and Al(OH)₃. **p* < 0.05, ***p* < 0.005, *** *p* < 0.0005. **a, b** Results are shown as averages with standard deviation. **a** Total serum IgE levels of BALB/c, SJL/j and C57BL/6 mice sensitized with pollen extract and Al(OH)₃. BALB/c mice produced significantly more IgE with or without alum than SJL/j or C57BL/6 mice. **b** Time course of IgE production in BALB/c and SJL/j mice sensitized with pollen extract and Al(OH)₃. Significant differences in IgE levels are seen after the 4th and 5th sensitization. **c** Individual values and means (solid bar) of IgE levels of BALB/c and SJL/j mice sensitized 5 and 6 times with PE and Al(OH)₃. In both mouse strains, the rise in IgE levels from the 5th to the 6th sensitization was significant.



in Western blots of purified natural Phl p 1, confirming the observation that only SJL/j mice had produced reactive IgE antibodies against this particular allergen (fig. 3b). The specific IgE binding frequencies are summarized in table 1: Phl p 5a and 5b have the highest binding frequencies across all three mouse strains, followed by the protein bands containing Phl p 4 and 13.

Total Serum IgE Levels in Mice Sensitized with Pollen Extract w/o Al(OH)₃

To investigate the effects of Al(OH)₃, the sensitization experiments were repeated in the absence of this adjuvant. In addition, F1 offspring from crosses between C57BL/6 × SJL/j (B6SJLF1/J) and BALB/c × SJL/j (CSLJF1/J) were included in the experiments. After the 5th sensitization, total serum IgE levels were lower than in

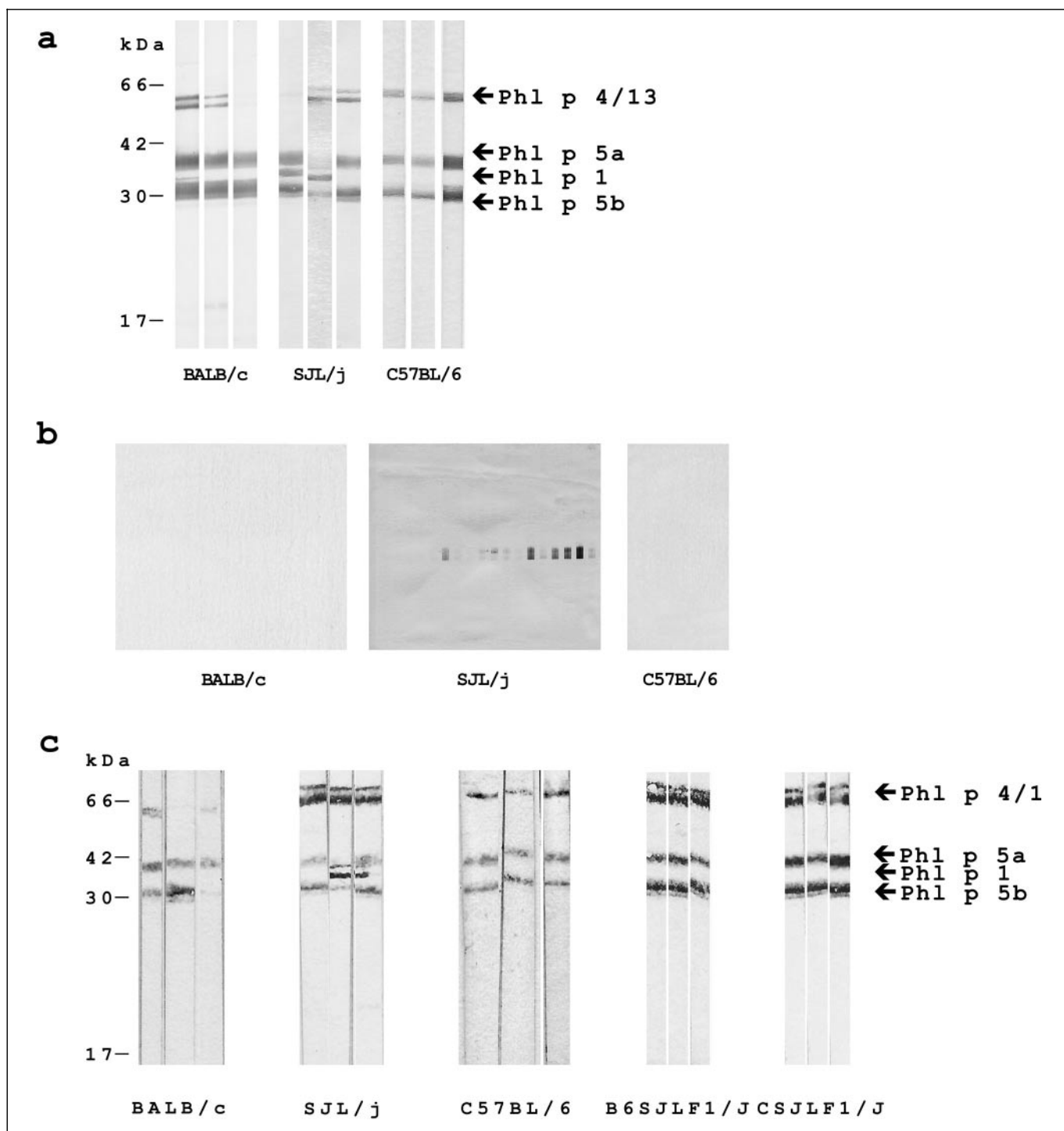


Fig. 3. Specific IgE reactivity of different mouse strains sensitized with *P. pratense* pollen extract with or without $\text{Al}(\text{OH})_3$. **a** Specific IgE reactivity against pollen extract is shown exemplarily for 3 mice of each strain (BALB/c, SJL/j and C57BL/6) sensitized with PE and $\text{Al}(\text{OH})_3$. The specific IgE reactivities were largely similar with respect to Phl p 5a, 5b, 4 and 13. Only SJL/j mice exhibited IgE reactivity against Phl p 1. **b** Specific IgE reactivity against natural Phl p 1. Sera of mice (BALB/c: n = 20; C57BL/6: n = 4, and SJL/j: n = 18)

were analyzed regarding their IgE reactivity to Phl p 1 in Western blots. Only the sera of SJL/j mice showed IgE reactivity to this allergen in this exemplary blot. **c** Specific IgE reactivity of different mouse strains sensitized five times with PE w/o $\text{Al}(\text{OH})_3$. Results are shown exemplarily for 3 mice of the strains investigated (BALB/c, SJL/j, C57BL/6, B6SJLF1/J and CSJLF1/J), respectively. The specific IgE reactivities are largely similar with respect to Phl p 5a and 5b. Only SJL/j mice produced specific IgE against Phl p 1.

Table 1. Specific IgE reactivity patterns and binding frequencies of sera from different mouse strains sensitized with *P. pratense* pollen extract

Major allergen	With Al(OH) ₃			Without Al(OH) ₃				
	BALB/c	SJL/j	C57BL/6	BALB/c	SJL/j	C57BL/6	B6SJLF1/J	CSJLF1/J
Phl p 1	0/40	13/22	0/4	0/10	2/5	0/8	0/10	0/10
Phl p 5a	40/40	18/22	4/4	10/10	5/5	7/8	10/10	10/10
Phl p 5b	40/40	18/22	4/4	10/10	5/5	8/8	10/10	10/10
Phl p 4/13	33/40	21/22	4/4	8/10	5/5	7/8	10/10	10/10

After five immunizations with pollen extract, IgE reactivity against the different major allergens was analyzed by Western blot.

the alum-treated animals; nonetheless the increases were significant ($p < 0.0005$) in all mouse strains except SJL/j and C57BL/6 (fig. 4a). Interestingly, IgE serum levels in CSJLF1/J mice were the highest, being significantly higher in comparison to all other strains investigated. The B6SJLF1/J and BALB/c mice exhibited intermediately increased IgE levels, and of these two strains only B6SJLF1/J mice showed significantly higher levels than SJL/j mice.

Pollen-Extract-Specific Immunoglobulin Isotypes in Mice Sensitized with Pollen Extract w/o Al(OH)₃

The analysis of anti-pollen extract-specific antibodies of different isotypes using an antigen-specific ELISA showed that sensitization with pollen extract induced the production of specific IgG1, IgG2a and IgG2b antibodies in all mouse strains investigated (fig. 4b). All increases in pollen extract-specific antibody isotypes IgG1, IgG2a and IgG2b were significant ($p < 0.05$).

The most striking observation was that at the same serum dilutions CSJLF1/J mice produced significantly higher levels of PE-specific IgG1 than all other strains investigated except B6SJLF1/J mice ($p < 0.05$). B6SJLF1/J mice also produced significantly higher levels of specific IgG1 than BALB/c and C57BL/6 mice ($p < 0.05$).

The results of pollen-extract-specific IgE antibodies are shown in figure 4c. Statistical analysis revealed that all mice produced significant levels of specific IgE antibodies, even SJL/j mice ($p < 0.05$). Moreover, in BALB/c mice specific IgE levels were significantly higher than in all other mouse strains ($p < 0.05$), and C57BL/6 mice also produced more of this specific immunoglobulin than SJL/j mice ($p < 0.05$).

Table 2. ICHR of different mouse strains sensitized with pollen extract without alum and challenged with PE or Phl p 1

Strain (sensitization; challenge and intradermal treatment; n)	ICHR
BALB/c (PE; PE; n = 3)	+
BALB/c (PE; Phl p 1; n = 4)	Ø
SJL/j (PE; PE; n = 2)	+
SJL/j (PE; Phl p 1; n = 2)	+
C57BL/6 (PE; PE; n = 3)	+
C57BL/6 (PE; Phl p 1; n = 3)	Ø
B6SJLF1/J (PE; PE; n = 3)	+
B6SJLF1/J (PE; Phl p 1; n = 2)	Ø
CSJLF1/J (PE; PE; n = 2)	+
CSJLF1/J (PE; Phl p 1; n = 3)	Ø

Mice were sensitized five times, challenged once intranasally and treated intradermally as indicated. ICHR was determined as described in the Materials and Methods. Ø = No ICHR response; + = positive ICHR response. All negative controls were negative, and positive controls were positive.

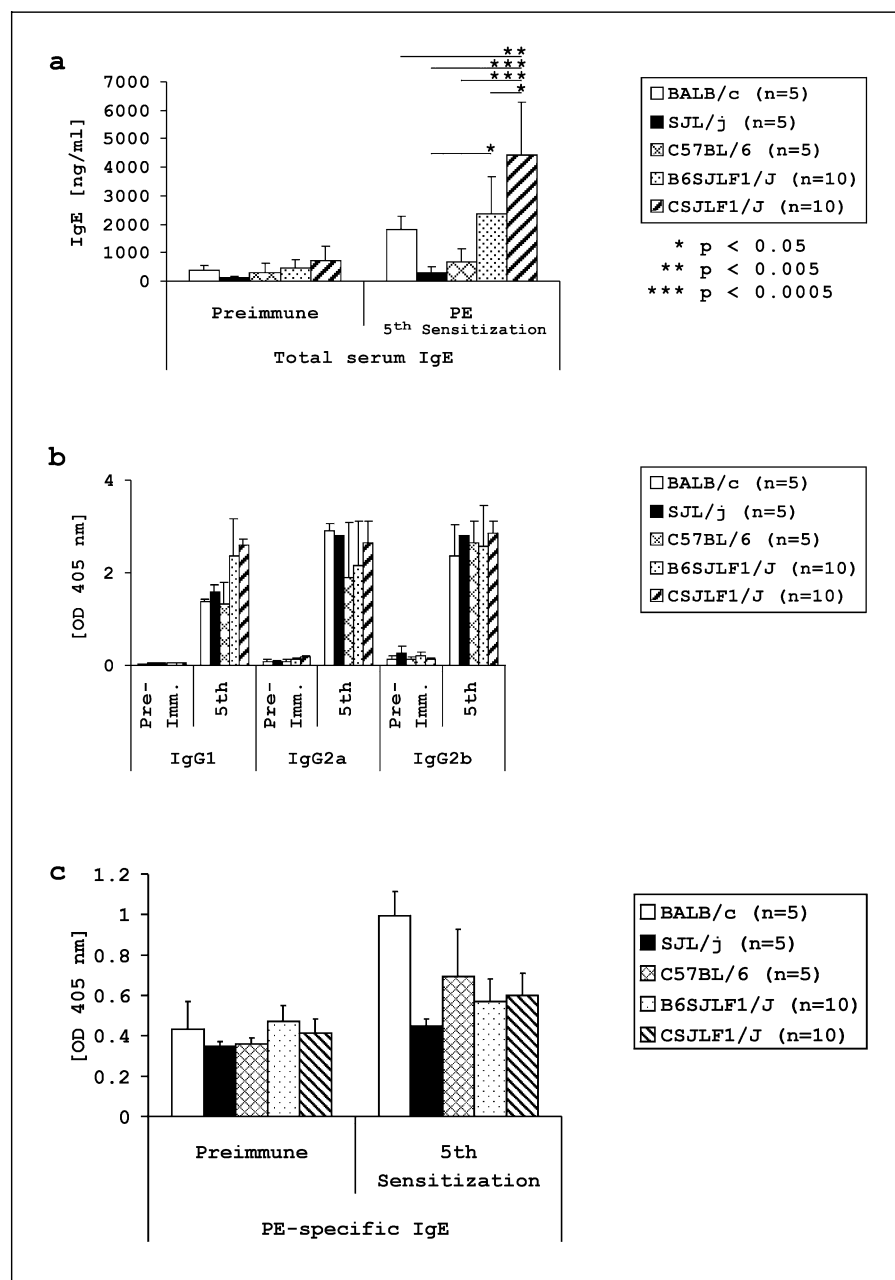
Specific IgE Reactivity to Major Allergens of Mice Sensitized with Pollen Extract w/o Al(OH)₃

The results of specific IgE reactivity to major allergens of PE are shown in figure 3c and are summarized in table 1. In all strains, IgE reactivity patterns were largely similar with respect to Phl p 5a and 5b. Only SJL/j mice mounted a specific IgE response against Phl p 1.

Immediate Cutaneous Hypersensitivity in Pollen-Extract-Sensitized Mice w/o Al(OH)₃

The results of the skin test of pollen extract-sensitized mouse strains is summarized in table 2. The PE-sensi-

Fig. 4. Total IgE serum levels and specific immunoglobulin levels in different mouse strains sensitized with *P. pratense* pollen extract w/o Al(OH)₃. Results are shown as averages with standard deviation. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. **a** Total serum IgE levels in mouse strains sensitized with *P. pratense* pollen extract w/o Al(OH)₃. **b** Pollen extract-specific immunoglobulin levels of the IgG1, IgG2a and IgG2b subclasses. **c** Pollen extract-specific IgE levels.



tized mice of all strains investigated showed a wheal-and-flare reaction after intranasal challenge with and intradermal application of the allergen. Only SJL/j mice shown to produce Phl p 1-specific IgE reacted positively upon challenge with natural purified Phl p 1 protein. Control mice were consistently negative and only showed an ICHR with the positive control.

Discussion

All mice examined in the present study (BALB/c, C57BL/6 and SJL/j) sensitized systemically with pollen extract produced high levels of IgE. Optimal responses were induced when this allergen was precipitated with alum as an adjuvant. However, the adjuvant alone also provoked the production of non-specific IgE, an observa-

tion which has previously been shown in other animal models [15, 16]. Thus, alum mainly affected the synthesis of total serum IgE, with the relative levels of production in the mouse strains remaining the same with or without alum (BALB/c > C57/BL6 > SJL/j). Interestingly, even though the total IgE response to PE in SJL/J mice was very low in comparison to that of BALB/c and C57BL/6, which is in accordance with other published data [7, 13, 17], SJL/j mice were capable of producing IgE levels after the 6th sensitization which were comparable with levels in BALB/c mice after the 5th sensitization, thus making their classification as low responders debatable, although BALB/c mice indeed still produced considerably higher levels after the 6th sensitization. In a study investigating IgE responses in BALB/c and C56BL/6 to a recombinant mosquito salivary allergen, it could also be shown that both strains produce IgE in the absence of alum as an adjuvant, but BALB/c mice also produced significantly more IgE [18]. Despite this difference and as shown by our study, both strains developed a positive immediate skin reaction. A further study investigated strain differences in the induction of allergic rhinitis without adjuvant, also showing the induction of IgE in BALB/c and C57BL/6 mice [19]. SJL/j mice on the other hand are generally considered to be low IgE responders due to a failure in early IL-4 generation [13]; this low IgE responsiveness in SJL/j mice is not due to a defect in IL-4 production per se, but may be due to an increased IFN- γ production by CD8+ T cells which inhibits class switch and suppresses IgE antibody production in these mice [20]. We could demonstrate that SJL/j mice not only mounted an IgE response but also showed a skin reaction upon intradermal inoculation of pollen extract, which is in contrast to findings by other groups where SJL/j mice were neither found to produce IgE [21] nor exhibited a skin reaction [7]. We suggest that the use of alum in investigations concerning sensitization to the major allergens of *P. pratense* pollen in mouse models should be well considered, since measurable levels were also obtained in experiments without alum. Although we cannot rule out that the extract itself may contain components that have adjuvant activity promoting a sensitization process, the avoidance of further adjuvant effects would still more closely resemble the clinical situation of allergic sensitization to an allergen.

Western blot analysis and ELISA showed that all the mouse strains investigated as well as the F1 progeny used produced PE-specific antibodies of the IgE, IgG1, IgG2a and IgG2b subclasses upon sensitization without alum. The IgG1 immunoglobulin levels were highest in CSJLF1/J mice, and no significant differences in the lev-

els of IgG2a and IgG2b subclasses were seen. Since the production of the two antibody isotypes IgE and IgG1 reflect the induction of a TH2 response in mice, it is apparent that this type of immune response can be induced by PE, even in the low-responder SJL mice.

A surprising observation was the inability of PE-sensitized BALB/c, C57BL/6 and also AJ mice (data for the latter not shown) to produce IgE antibodies and to exhibit an ICHR against Phl p 1, which represents one of the most important components of timothy grass. Only PE-sensitized SJL/j mice produced Phl p 1-specific IgE and showed an ICHR against this allergen. Since neither the F1 generations of BALB/c $\times$ SJL/j nor C57/BL6 $\times$ SJL/j mounted an IgE response against Phl p 1, a dominant genetic component appears to be involved in the IgE reactivity to Phl p 1.

Differing immune reactions among various mouse strains are primarily attributable to differences in MHC genes, which may also explain the observations made in our study since all mouse strains differed in this respect. However, several observations suggest the involvement of other genetically linked loci to atopy-related phenotypes [22], such as the Tcrvb8, IL-4 and interferon- γ genes [23], the IL-5R α chain [24], IL-4R allotypes [25], and the differential regulation of antigen presentation in high and low IgE responder mice [26]. These aspects are to be considered in future investigations on the IgE reactivity to Phl p 1.

In conclusion, our study indicates that careful consideration should be given to the selection of a suitable mouse strain in attempts to develop models of human allergic disease and in the assessment of the inherent allergenic potential of proteins [27]. In addition, it is possible that further understanding of murine strain-related allergic reactions may assist in our understanding of the genetic basis of susceptibility to sensitization to allergens in humans.

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