

Sensitization to Inhaled Ryegrass Pollen by Collateral Priming in a Murine Model of Allergic Respiratory Disease

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Key Words

Mouse model • Allergy • Sensitization • Ryegrass •
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Abstract

Background: Mouse models of asthma suffer from the necessity to prime the animals by injections before respiratory exposure. Our aim was to develop a mouse model that mimics the progression of human allergic disease upon low-dose inhaled allergen exposure. **Methods:** Mice were primed intraperitoneally to ovalbumin (OVA) before they were exposed repeatedly to aerosols of either OVA, ryegrass (*Lolium perenne*) pollen extract, or both concomitantly. The sensitization to ryegrass pollen proteins was evaluated by measurement of specific serum antibody, by the respiratory response to a challenge with ryegrass pollen extract and by lung cytokine production after challenge. **Results:** Inhalation of ryegrass pollen extract alone did not result in sensitization. Sensitization to inhaled ryegrass pollen proteins, however, did occur in mice that had been sensitized to OVA by intraperitoneal injections and were then exposed to inhaled ryegrass pollen extract and OVA simultaneously. T and B cell priming was ascertained by ryegrass pollen-specific IgG1 and IgE antibody production and by induction of air-

way inflammation and of Th2 cytokine mRNA transcripts in the lungs upon airway challenge with ryegrass pollen extract. A progressive spread of the IgE/IgG1 response to different ryegrass pollen proteins could be visualized in immunoblots by comparing antibody patterns at day 56 and 86. **Conclusions:** Low-dose inhalatory allergen exposure results in sensitization when airways are exposed at the same time to another allergen to which the animals are already sensitized. This model can help to unravel the mechanisms that underlie the development and progression of respiratory allergic diseases.

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Introduction

Unraveling the mechanisms that induce sensitization to inhaled allergens and that perpetuate the allergic immune response is a major research issue, in view of the ascending slope of allergic asthma prevalence [1]. The reasons why only some subjects become sensitized to ubiquitous inhaled allergens remain ill understood. Studies on the mechanisms involved in allergic sensitization are hampered, in almost all animal models, by the requirement for intraperitoneal priming, often at high al-

Table 1. Description of the 4 groups of mice, differing by the inhaled allergen mixture for aerogenic sensitization

Group names	Intraperitoneal sensitization ^a	Aerogenic sensitization ^b	Ryegrass pollen challenge ^c
Lol	OVA	RPE 1 mg/ml	RPE 1 mg/ml
OVA	OVA	OVA 10 mg/ml	RPE 1 mg/ml
OVA+Lol	OVA	OVA 10 mg/ml + RPE 1 mg/ml	RPE 1 mg/ml
Control	Saline	OVA 10 mg/ml + RPE 1 mg/ml	RPE 1 mg/ml

RPE = Ryegrass (*L. perenne*) pollen extract.

^a Mice were sensitized to OVA by i.p. injection of 100 µg of OVA in alum on day 0 and 7 or sham sensitized with saline.

^b Aerosol exposure was a 5-day course of a daily 15-min aerosol exposure to different allergen mixtures on days 35–39, 49–53 and 63–67.

^c Ryegrass pollen challenge consisted of a 5-day course of a daily 15-min aerosol exposure to ryegrass pollen extract on days 77–83 and on day 86.

lergen doses and in association with adjuvants, before a response to respiratory inhaled allergen can be induced [2–4]. This contrasts with the situation in humans, in whom priming does not occur through systemic sensitization. Allergic airway disease in mice can also be induced without systemic sensitization, by repeated airway exposure to some specific allergens with protease activity, for example house dust mite Der p 1 [5, 6]. However, when a non-protease-associated allergen is used, like ovalbumin (OVA) [7, 8], sensitization through inhalation seems to be transient and is progressively replaced by immunological tolerance towards the inhaled antigen [9]. Moreover, aerosol exposure to OVA in nonprimed animals can induce a state of immunological unresponsiveness [10–14]. Another way to bypass systemic sensitization is to administer allergen in combination with endotoxins, suggesting that activation of airway dendritic cells is a prerequisite for sensitization [15]. Altogether, there is a need for mouse models that more closely mimic the human situation and that lead to progressive sensitization by inhalation of low doses of allergen.

Clinical observations suggest that sensitization to one allergen favors sensitization to other environmental allergens [16–18], a phenomenon called Th2 spread. Although the first report on the possibility to expand an immune response to an antigen through the presence of another antigen in the same microenvironment dates back to 1993 [19], most data on the possible mechanisms

that underlie this phenomenon are recent. It has been shown in a mouse model that Th2-type sensitization to one antigen can be transferred by inhalation to another antigen at the condition that both are given simultaneously during challenges [20, 21]. This mechanism of sensitization, called collateral priming, was found to be different from the mechanisms that underlie a primary sensitization [20]. Furthermore, it is the IL-4 produced by the already primed T cells that influences the Th2 polarization of the naïve T cells encountering a new antigen [20–22]. The phenomenon is thought to occur when two T cells with different specificities become activated in the same microenvironment, which might imply encounter with the same antigen-presenting dendritic cell [21]. In the present work, we intended to develop a mouse model of allergic asthma in which priming occurs after repeated aerosol exposure to low pollen allergen doses, as it occurs by repeated seasonal low-dose pollen exposure in allergic patients, and we explored whether the principle of collateral priming could induce such an airway sensitization.

Methods

Ryegrass Pollen Extract

Ryegrass (*Lolium perenne*) pollen (Allergon, Ängelholm, Sweden) extract was prepared in phosphate buffer pH 8.0, at room temperature for 3 h with continuous pH adjustment, as already described [23]. The extract was centrifuged (30 min, 3,500 g, 4°C), sterilized through a 0.45-µm membrane and kept at –20°C until use. Protein content was 2.8 mg/ml, as determined by the Coomassie blue assay (Bio-Rad, Richmond, Calif., USA).

Sensitization and Airway Challenges

Balb/c mice (males, 5 weeks old) were kept under specific pathogen-free conditions. Starting from day 1, mice were injected twice at a 1-week interval with 100 µg OVA in 150 µl saline + 50 µl alum (ImjectAlum™; Pierce) intraperitoneally (i.p.). From day 35 to 39, mice were exposed daily for 15 min to either aerosolized ryegrass pollen extract alone (1 mg/ml), OVA alone (10 mg/ml) or OVA (10 mg/ml) + ryegrass pollen extract (1 mg/ml). Concentrations of ryegrass pollen extract and OVA were determined on the basis of preliminary experiments. Aerosol exposure was repeated from day 49 to 53 and from day 63 to 67. A control group was defined as mice injected i.p. with saline and exposed by aerosol to OVA (10 mg/ml) + *Lolium* pollen extract (1 mg/ml). The 4 groups of mice are described in table 1. In order to assess the efficacy of OVA sensitization, OVA-specific IgG1 were measured by ELISA. High OVA-specific IgG1 antibody levels were measured in mice after i.p. OVA sensitization, but not in the control (saline) group (data not shown).

To evaluate T cell sensitization to ryegrass pollen proteins, mice of all groups were challenged from day 77 to 83 with ryegrass pollen extract (1 mg/ml). Metacholine (Mch) challenges were performed on day 84 with whole-body plethysmography or with the

forced oscillation technique (see below). On day 86, all groups of mice were challenged again with ryegrass pollen extract for 15 min before sacrifice 2–6 h later.

To obtain ryegrass-hyperimmunized serum, 5 mice were injected twice at a 2-week interval with 100 or 500 µg of ryegrass pollen proteins in alum (ImjectAlum). Blood samples were taken 2 weeks later and sera were pooled.

The study was approved by the ethics committee for animal experimentation of the Catholic University of Leuven.

Serum Levels of Specific Antibodies

Blood was taken at day 0, at day 56 (after the second series of aerosol exposures) and at day 86 (day of sacrifice). The serum levels of *Lolium*-specific IgG1 and IgE were measured by ELISA. Briefly, polystyrene microtiter plates (Maxisorb™; Nunc, Roskilde, Denmark) were coated overnight at 4°C with *Lolium* extract (10 µg/ml) in 0.02 M PBS, pH 8.0. Free binding sites were blocked with PBS-gelatin 2% before serum samples were added at the following concentrations: 1/3 for the detection of *Lolium*-specific IgE and 1/10,000 for IgG1. All dilutions were made in PBS-gelatin 0.5%. For IgE measurements, serum samples were first incubated with protein G-Sepharose (Amersham Biosciences, Roosendaal, The Netherlands) to deplete the samples from IgG. For that, 50 µl of gel slurry (1/1) was added to 100 µl of diluted serum sample and incubated for 30 min at room temperature with shaking. The IgG-depleted serum samples, now diluted 1/4, were recovered after sedimentation of the protein G-Sepharose gel. The binding of specific antibodies was further detected by incubation for 1 h at room temperature with biotinylated monoclonal anti-mouse IgG1 or IgE (BD Biosciences Pharmingen, San Diego, Calif., USA), respectively, followed by peroxidase-labeled streptavidin (Jackson ImmunoResearch Laboratories, West Grove, Penn., USA) for 1 h at room temperature. The colorimetric reaction occurring after addition of TMB in citrate buffer in the presence of hydrogen peroxide was read at 450 nm after addition of sulfuric acid solution. All washes were made with PBS-Tween-20 0.05%.

SDS-PAGE Immunoblotting

SDS-PAGE was carried out in 13% polyacrylamide separating gels with 5% polyacrylamide stacking gels. Before runs, ryegrass pollen extract was heated at 100°C in sample buffer (Tris 125 mM, pH 6.8, glycerol 17.5%, SDS 4%, β-mercaptoethanol 1% and bromophenol blue 0.002%), before application into the gel (30 µg of protein/cm). After the run, proteins were blotted onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, Mass., USA) by electrotransfer. The blotted membrane was then blocked in PBS-skimmed milk 0.2%. Strips were cut and incubated overnight at 4°C in mouse serum (8 µl/strip for IgG1 detection or 125 µl/strip for IgE detection) diluted in PBS-skimmed milk 0.1%. Bound mouse antibodies were detected by biotinylated monoclonal anti-mouse IgG1 or anti-mouse IgE (BD Biosciences Pharmingen, San Diego, Calif., USA), respectively, both 1/1,000 in PBS-BSA 0.5%, followed by peroxidase-labeled Extravidin (Sigma, St. Louis, Mo., USA). Bands were revealed by incubation in TMB (KPL, Gaithersburg, Md., USA).

Measurement of Bronchial Hyperresponsiveness

Whole-Body Plethysmography

On day 84, that is, 24 h after aerosol challenge with ryegrass extract, airway responsiveness was evaluated by Mch-induced

airflow obstruction in mice individually placed in a whole-body plethysmograph (Buxco™; EMKA Technologies, Paris, France). Airway responsiveness was reflected by the value of enhanced pause (P_{enh}), which correlates with the degree of airway resistance [24]. Mice were first exposed to nebulized saline, to determine the P_{enh} baseline, then to incremental doses of nebulized Mch (0, 5 and 10 mg/ml). Each nebulization was given for 1 min, and the P_{enh} was consecutively recorded every 30 s for 3 min, which allowed us to calculate the mean P_{enh} for every mouse in every condition.

Forced Oscillation Technique

Alternatively, in separate groups of mice, airway responses to inhaled Mch were measured by the forced oscillation technique. (FlexiVent™; Scireq, Montreal, Que., Canada). Mice were anesthetized with sodium pentobarbital (70 mg/kg i.p.) and immobilized. An 18-gauge metal catheter was placed in the trachea of immobilized mice and connected to a computer-driven ventilator. The animals were sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O, to achieve a mean lung volume close to that of spontaneous breathing. After baseline measurement, mice were challenged with increasing concentrations of Mch (0, 0.625, 1.25, 2.5 and 10 mg/ml) generated with an in-line nebulizer and administered through the ventilator. Airway resistance was measured by using a 'snapshot' protocol (each 20 s during 2 min). For each Mch concentration, the mean of 6 measurements was calculated.

Histology and Quantitative PCR on Lung Tissue

At day 86, mice underwent a 'provocation test', that is, a single aerosol exposition of ryegrass pollen extract, before they were sacrificed between 2 and 6 h later. Pieces of lungs were taken from the mice, immediately frozen in liquid nitrogen and kept at -80°C until use. Total RNA was extracted from homogenized tissue samples by using an RNeasy minikit (Qiagen Benelux, Venlo, The Netherlands). Next, poly-A RNA was transcribed into cDNA by using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif., USA). The cDNA samples were then used for real-time quantitative PCR, performed in the ABIprism 7700 sequence detector (Applied Biosystems, Foster City, Calif., USA), as previously described [25]. The sequences of the primers and probes for IL-4, IL-5, IL-13 and β-actin, purchased from Eurogentec (Seraing, Belgium), have been previously reported [25]. Each PCR amplification was performed in triplicate, using the following conditions: 95°C for 10 min, followed by 40 cycles consisting of 95°C for 15 s and 60°C for 1 min. To quantify the target gene in the unknown samples, we used cDNA plasmid standards specific for each individual target, as already described [25]. All results were normalized to β-actin to compensate for differences in starting cDNA amounts.

For histopathology analysis, lung pieces were fixed and kept in formaldehyde 6% until use. They were then embedded in paraffin with a standard protocol, and 6-µm slices were made. After deparaffinization and rehydration, the slices were hematoxylin/eosin-stained and analyzed by light microscopy. The maximal thickness of randomly selected inflammatory infiltrates in the lung slices were measured using an eyepiece graticule (10 measurements per group).

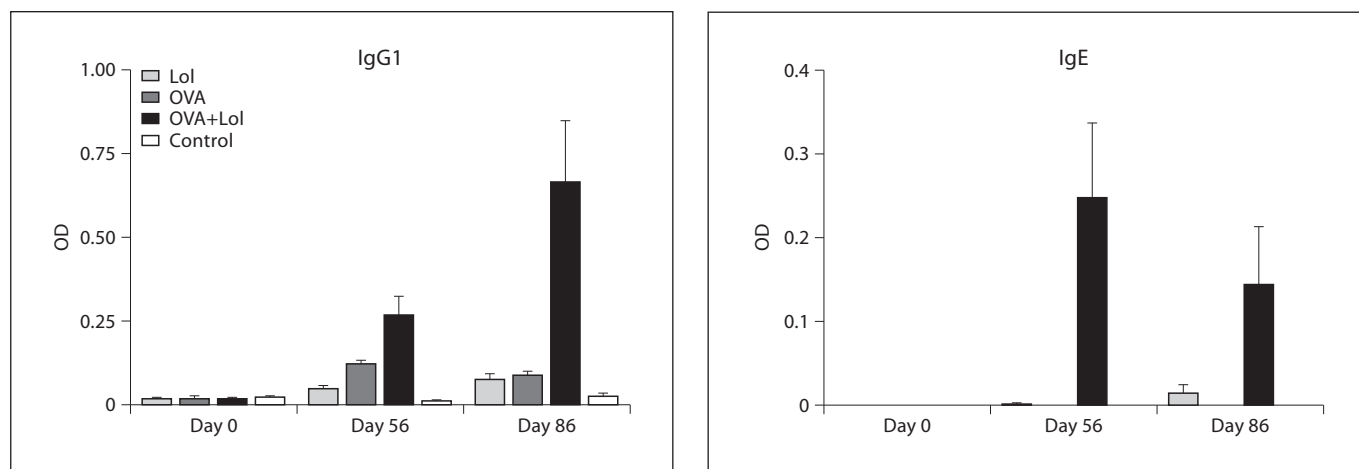


Fig. 1. Antibody responses (IgG1 and IgE) to ryegrass proteins as measured by ELISA on days 56 and 86. Results are expressed in ELISA units. The 4 groups were defined on the basis of the protocol for inhalation and sensitization (table 1).

Statistical Analyses

When appropriate, the groups were compared by using the Kruskal-Wallis test. Selected Dunn's posttest was used to compare the OVA + *Lolium* (OVA+Lol) group with the other groups.

Results

Mice were first sensitized intraperitoneally with OVA (or sham sensitized with saline) before they were subjected to 3 episodes (each lasting 5 days) of daily aerosol exposure with OVA alone, ryegrass pollen extract alone, or with ryegrass pollen extract and OVA together. Thereafter, mice of all groups were daily challenged for 5 days with ryegrass pollen extract alone (without OVA) from day 77 to 83 and again (before sacrifice) on day 86. The different groups are described in table 1.

Serum Levels of Specific Antibodies

In figure 1, we show the serum levels of ryegrass pollen-specific antibodies on days 56 and 86. High levels of specific IgG1 and IgE were produced by the mice that inhaled ryegrass pollen extract together with OVA, but only if they had previously been sensitized to OVA by intraperitoneal injections. The IgG1 and IgE anti-ryegrass pollen antibody levels with this sensitization method represented approximately 42 and 17% of the levels obtained in ryegrass pollen-hyperimmunized mice. Taken together, those results indicate that a Th2-type response was raised against ryegrass pollen proteins exclusively in the

mice that inhaled ryegrass pollen extract together with OVA, and that had previously been sensitized to OVA.

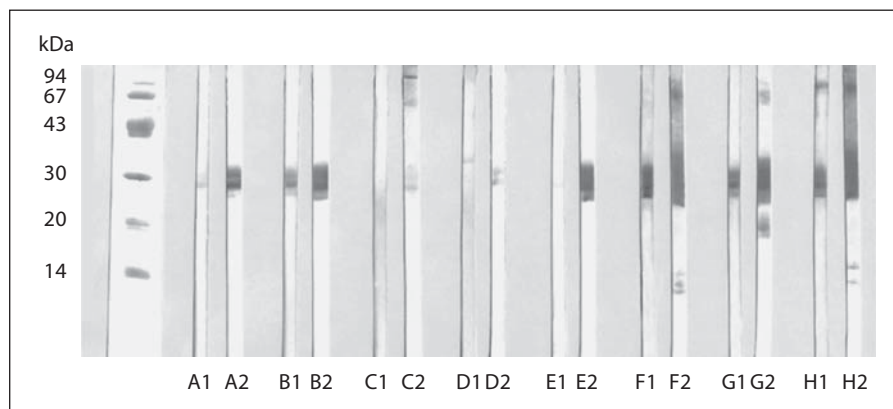
Specificity of the Antibodies

We performed SDS-PAGE immunoblotting in order to detect the ryegrass pollen antigens towards which the mice of the OVA+Lol group produced IgG1 and IgE antibodies. In figure 2, we compare the IgG1 patterns at day 56 and 86 in 8 individual mice. The immune response started generally against a 'major' band at 30 kDa as seen on day 56, before spreading to additional 'minor' antigen bands at 14, 16, 18, 60 and 100 kDa, as can be seen at day 86. It must be noted that, besides the bands at 30 kDa, no allergen has yet been described at such molecular masses in ryegrass pollen. In figure 3, we investigated the specificity of the IgE response. We could detect IgE antibodies in 5/6 mice of the OVA+Lol group. The IgE response was directed only towards the 'major' antigen, represented by the 2 bands at around 30 kDa. When comparing the IgG1 patterns with the IgE patterns of the same mice, it appears that the intensity of the IgE response towards the 30-kDa allergen correlated with the intensity of the IgG1 response towards that same antigen.

Airway Hyperreactivity

In order to examine whether mice were reactive to inhaled ryegrass, we assessed on day 84 the effects of aerosol exposure to ryegrass pollen extract (challenge episode) on airway hyperreactivity. First, we used whole-body plethysmography to measure Mch-induced airflow obstruction in

Fig. 2. SDS-PAGE immunoblot showing serum IgG1-binding patterns to ryegrass pollen proteins of 8 mice in the OVA+Lol group, at day 56 (A1–H1) and at day 86 (A2–H2). Molecular mass markers are shown on the left side. There was no detectable IgG1 on immunoblots in the other groups.



the different groups of mice at day 84. Figure 4 shows that the OVA+Lol group was more reactive to Mch than the Lol group and the OVA group. The higher airway hyperreactivity in the former group was apparent at the dose of 5 and 10 mg/ml of Mch. There was no significant difference between the OVA+Lol group and the OVA group at the Mch dose of 20 mg/ml, although a trend towards higher reactivity was still evident in the OVA+Lol group.

In addition to whole-body plethysmography, we used the forced oscillation technique to measure airway resistance (fig. 5). Again, we found that the OVA+Lol group was significantly more reactive than the Lol group, but it did not differ significantly from the OVA group in this experiment.

Histology and Quantitative Expression of Cytokine Transcripts

In order to evaluate T cell sensitization to ryegrass pollen proteins, on day 86, mice of all groups underwent another aerosol challenge with ryegrass pollen extract for 15 min. Lungs were taken between 2 and 6 h later. Analyses of the lung slices (fig. 6a–c) demonstrated a severe pulmonary inflammation in the OVA+Lol group, with massive cell infiltration, whereas no inflammation was apparent in the lungs of the Lol group. In the OVA group some inflammation was also visible by microscopy, probably as a result of residual OVA-induced inflammation, 2 weeks after the last exposure to aerosolized OVA. The thickness of inflammatory cell infiltrates was measured in the different groups to obtain a semiquantitative evaluation (fig. 6d) and the largest infiltrates were present in the OVA+Lol group.

Gene expression of IL-4, IL-5 and IL-13 in the lungs, 2–6 h after ryegrass exposure, was evaluated by quantitative RT-PCR. Figure 7 shows that IL-4 and IL-13 gene

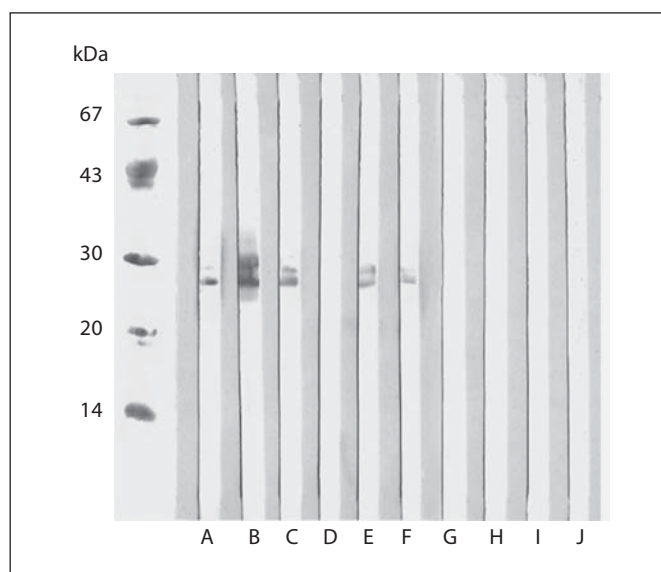


Fig. 3. SDS-PAGE immunoblot showing the IgE-binding patterns to ryegrass pollen proteins of 6 mice in the OVA+Lol (A–F) group, and from the OVA (G, H) and Lol (I, J) groups. Molecular mass markers are shown on the left side.

transcription was exclusively induced in the OVA+Lol group. However, a high IL-5 mRNA production was also seen in the OVA group, which we attribute to the residual OVA-induced airway inflammation. Taken together, those data are further evidence that the mice of the OVA+Lol group had developed a Th2-biased immune response against ryegrass pollen proteins.

Control Experiments

We carried out additional experiments in which we measured the sensitization to ryegrass pollen proteins af-

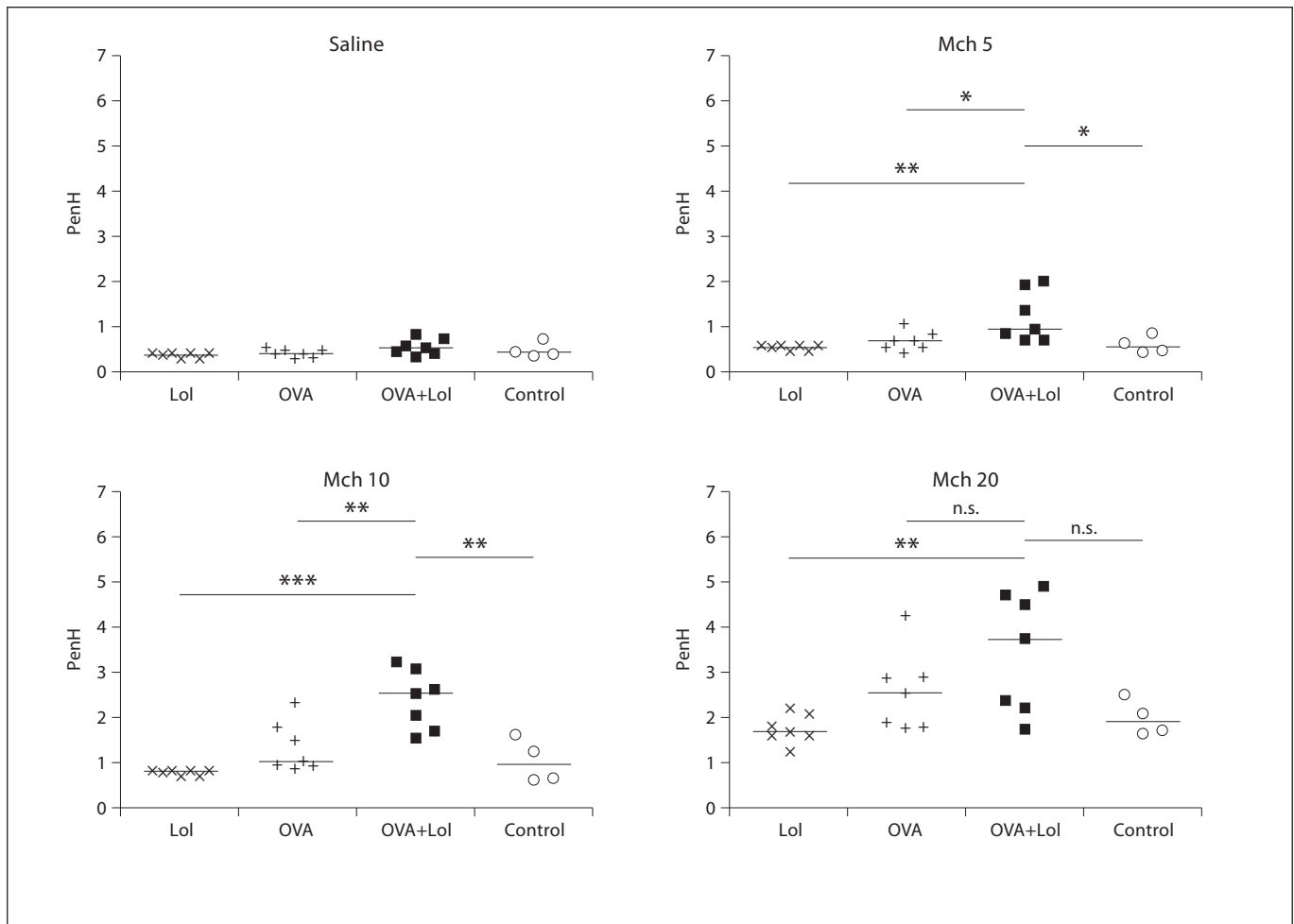
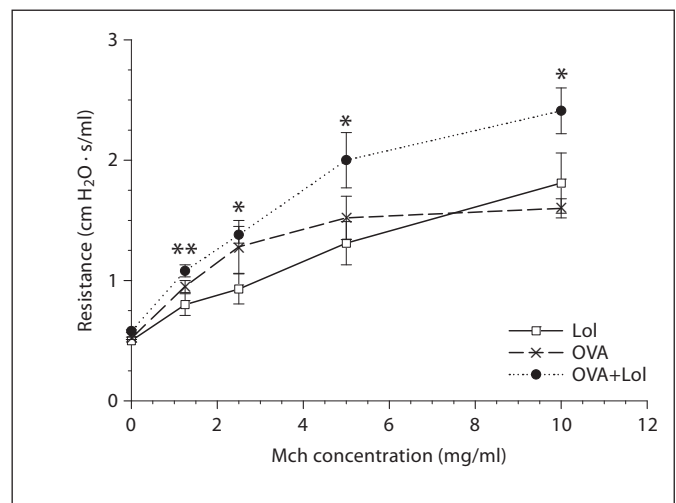


Fig. 4. Airway hyperreactivity to Mch. Bronchial responsiveness to incremental doses (5–20 mg/ml) of Mch was measured using barometric whole-body plethysmography at day 84. One point represents one mouse. Statistical analysis by Kruskal-Wallis test, followed by Dunn's post test. n.s. = Not significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 5. Airway resistance, as measured at day 84 with forced oscillation technique in response to incremental doses of Mch (1.25–10 mg/ml). Statistical analysis by Kruskal-Wallis test at each Mch concentration (* $p < 0.05$; ** $p < 0.01$). Dunn's post test was applied as Kruskal-Wallis test was significant: OVA vs. OVA+Lol, $p < 0.01$ at 1.25 mg/ml of Mch; Lol vs. OVA+Lol, $p < 0.05$ at 2.5 and 5 mg/ml.



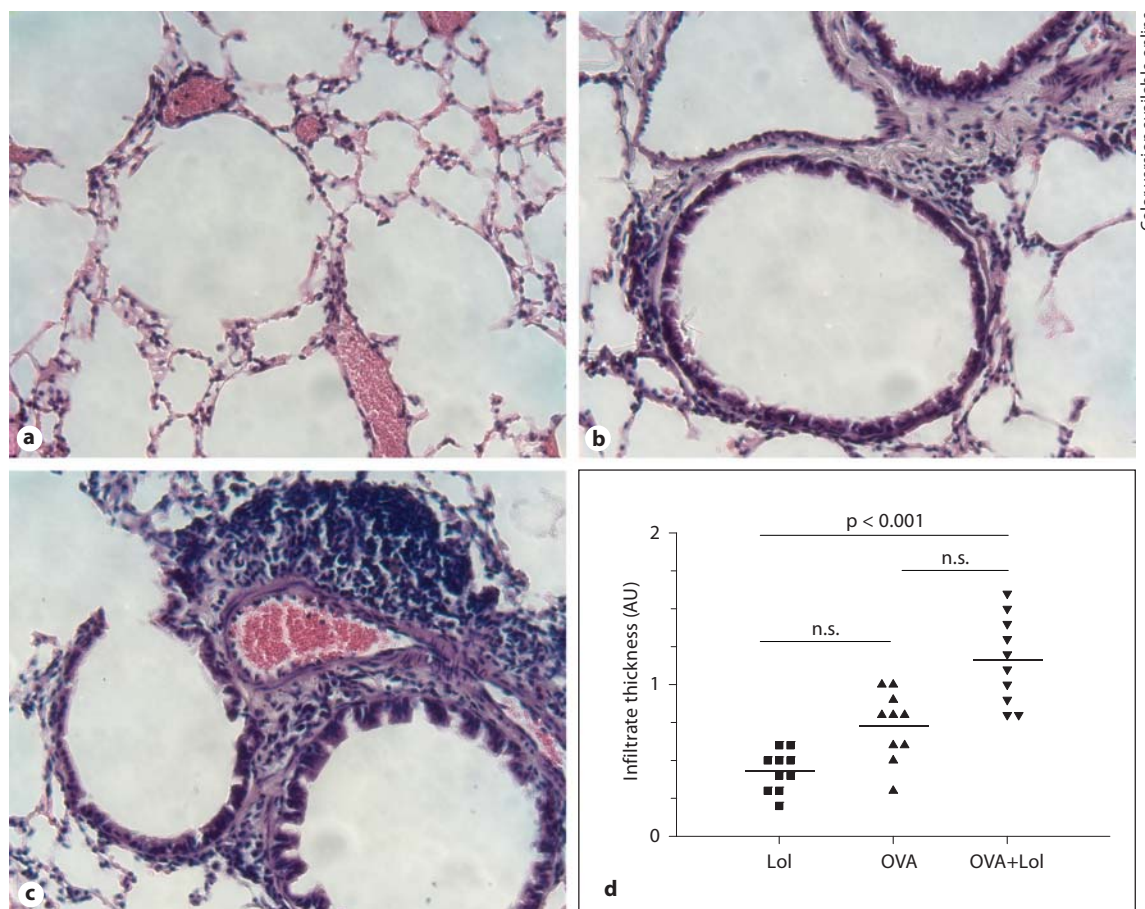


Fig. 6. Lung histology (a–c) and thickness of the inflammatory infiltrates (d). Slices of lung tissue were made on day 86, after exposure to Lol pollen proteins. Large inflammatory cell infiltrates are visible in the OVA+Lol group. The maximal thickness of the inflammatory infiltrates was measured microscopically in each group, expressed in arbitrary unit corresponding to graticule grid (d). Kruskal-Wallis: $p < 0.0001$, followed by Dunn's post test. Horizontal bar is the mean for each group.

ter only 1 episode of co-inhaled OVA-ryegrass pollen extract in OVA-primed mice. We found that this was not enough to induce sensitization towards ryegrass proteins (data not shown). Likewise, 3 co-exposures of OVA-ryegrass pollen extract at a lower concentration (0.1 mg/ml) were not able to induce sensitization to ryegrass pollen proteins (data not shown). This indicates that a sufficient dose and number of exposures to co-inhaled antigens are needed to induce sensitization.

Discussion

In this paper, we intended to set up a mouse model of Th2 sensitization to an inhaled allergen, without intraperitoneal priming, thus mimicking more closely the in

vivo human situation. We first systemically sensitized mice to OVA by intraperitoneal injections with alum. Such Th2 response type-inducing sensitization procedure, when followed by exposure to aerosolized OVA, is known to provoke respiratory allergic inflammation and hyperreactivity to Mch [2, 3]. Sensitization to OVA was ascertained by OVA-specific antibody titration. Thereafter, we exposed the mice to several aerosol episodes with OVA alone, ryegrass (*L. perenne*) pollen extract alone, or OVA mixed with ryegrass pollen extract. Thereafter, B and T cell sensitization to ryegrass pollen was studied.

The most striking observation in our work is that OVA-primed mice become gradually sensitized to ryegrass pollen proteins during repeated inhalation of a mixture of OVA and ryegrass pollen extract (referred to as OVA+Lol group). Sensitization, however, did not occur

out the use of a Th2-skewing adjuvant. Sensitization to ryegrass pollen did not occur in any of the other experimental groups. Some other studies also achieved sensitization to co-administered antigens after only 1 or 2 challenges. They differ from our study by the fact that the antigens were given intranasally and in high amounts [20, 21, 26]. In the one study most comparable to ours [21], 100 µg of ragweed protein was given intranasally to OVA-primed mice, together with 20 µg of OVA, in order to induce Th2 spread to the ragweed proteins. This is certainly much more than what our mice inhaled during one challenge, although it is difficult to estimate the exact amount of antigen deposited in the airways when using an aerosol. Fifteen minutes of exposure requires 5 ml of extract, and 10 mice are in the aerosol cage at the same time, which means that 500 µg of ryegrass protein should be available per mouse. However, huge amounts of antigen are lost with such a system and never reach the airways of the mice. When those data are taken together, we can reasonably think that very low doses of allergens reached the airways of the animals. In this regard, our model of Th2 spread mimics more closely the progression of allergic disease in humans, since exposure to pollen proteins at any given time will always be low. Finally, in a study by Goplen et al. [26], simultaneous inhalation of different allergens could induce sensitization. The underlying mechanism is not clear, but the possibility of adjuvant effects from endotoxins or other TLR ligands in the mixture has to be considered.

Given that mice were first sensitized and then exposed to OVA, with local inflammatory reactions as a consequence, we attempted to define whether such local OVA-induced inflammation could be held responsible for the sensitization to ryegrass. For that, we performed experiments in which ryegrass exposure occurred 24 h after OVA exposure, a delay which is not sufficient to see the resolution of the inflammatory infiltrate. No sensitization to ryegrass pollen proteins then was observed [unpubl. observation]. This suggests that the primary and secondary allergen must be given simultaneously, and that sensitization to the secondary allergen is not a mere consequence of the inflammation induced by the primary one. This observation corroborates the finding by others that copresentation of the secondary and the first antigen(s) by the same dendritic cell is the mechanism by which Th2 spread occurs [21].

In most mice, sensitization started towards the same 30-kDa antigen. Two episodes of exposure later, the mice had developed new IgG1 antibody specificities to other antigens within the ryegrass pollen protein mixture. The

IgE response in contrast was still restricted to the 30-kDa antigen, but the amount of serum IgE was low in these mice and one may consider that other IgE specificities, even if present, might have been undetectable. It was at first sight amazing that mice with the same genetic background recognized and mounted immune responses to different proteins of the antigen mixture, despite identical conditions of sensitization. Our immunoblot data, together with the knowledge on the mechanisms of Th2 spread as described by Hayashi et al. [21], suggest that spreading occurs at random, depending on the peptides that are copresented at the dendritic cell level, although this remains to be firmly established. The fact that most mice mounted first an immune response to the 30-kDa antigen might relate to the relative amount of that antigen within the ryegrass protein mixture. The higher the amount, the higher was the probability that it was copresented with OVA.

In our model, respiratory sensitization still depends on a primary sensitization to a non-pollen-related antigen. In allergic patients, if the mechanism of Th2 spread can explain the progressive sensitization to additional allergens, the question remains as how the first sensitization towards pollen allergens occurs. Based on the observation that food allergy in infancy is a good predictor of respiratory allergy at an older age, we suggest that at least in some cases the primary allergen is a food allergen. Through cross-reactivity with one of the pollen allergens, this food allergen is the substrate on which sensitization to pollen develops. Of note, a single cross-reactive epitope might even be sufficient as the starting point for sensitization to an additional allergen source [27], through a mechanism of what might be called intermolecular Th2 spread. This theory needs to be confirmed, and we believe that our model will be helpful in that respect.

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