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Rabbit IgG Directed to a Synthetic C-Terminal Peptide of the Major Grass Pollen Allergen *Lol p I* Inhibits Human Basophil Histamine Release Induced by Natural *Lol p I*

Key Words

Blocking antibodies
Grass pollen allergen *Lol p I*
Histamine release
Immunotherapy
Synthetic peptides

Abstract

The potential role of allergen-specific IgG antibodies as 'blocking' antibodies in allergen-induced human basophil histamine release was investigated. This was studied in a model with the major grass pollen allergen *Lol p I* and polyclonal rabbit antisera directed against this allergen and against a synthetic peptide of its C terminus. When allergen and antibodies were allowed to preincubate, *Lol p I* induced histamine release was inhibited up to 85% by the antiserum against *Lol p I*. By omitting preincubation, and thereby more closely mimicking an in vivo situation, up to 55% inhibition was realized. This indicates that allergen-specific IgG can act as 'blocking' antibody without preincubation. Immunization of rabbits with a synthetic C-terminal peptide of *Lol p I* resulted in antibodies reactive with natural *Lol p I*. Despite their 100-fold lower avidity for *Lol p I* (as compared with antinatural *Lol p I*), these antibodies had the capacity to inhibit *Lol p I* induced histamine release for >90% (up to 50% without preincubation). This indicates that it is possible to block histamine release induced by a major allergen with low-avidity IgG antibodies directed against a minor proportion of the allergen (25 amino acids). IgE antibodies from the donors studied were unreactive with this synthetic peptide, indicating that for blocking activity identical epitope specificity of IgE and IgG is not essential. This opens interesting perspectives for application of synthetic peptides in immunotherapy, distinct from their effects on T cell reactivity.

Introduction

Immunotherapy is widely accepted as an efficient treatment of allergic rhinitis [1, 2]. The mechanism by which immunotherapy exerts its beneficial effect is still largely unknown. The classical view that IgE-mediated responses are

prevented by 'blocking' IgG4 antibodies is now generally believed to be too simple [3]. Much attention is currently given to the influence of immunotherapy on T cells. The beneficial effect of immunotherapy has been linked predominantly to the late-phase allergic responses which were shown to be inhibited [4–9]. Recent reports have demon-

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strated an infiltration of T_H2 -type cells at the site of late-phase inflammatory reactions [9–13]. For the inhibitory effect of immunotherapy on late-phase responses, several mechanisms have been proposed, including a shift in the T_H1/T_H2 ratio in favor of T_H1 [9] and induction of allergen-specific CD8⁺ T suppressor cells [14–16]. Interestingly, Varney et al. [9] confirmed earlier observations [17–22] that not only the late-phase skin response is decreased during the course of immunotherapy, but also the early-phase response. In contrast to the late-phase response, where inflammatory T cells and eosinophils play a key role, the early-phase skin reactivity is mediated by sensitized mast cells without direct involvement of inflammatory T cells. An inhibitory effect of immunotherapy can, therefore, not be explained by changes at the T cell level. Decreased specific IgE levels were only reported after prolonged treatment, long after therapy started showing its beneficial effect [23, 24]. An alternative explanation could be allergen-specific IgG4 present in the intradermal tissue, acting as ‘blocking’ antibodies. Lack of correlation between clinical improvement and allergen-specific IgG4 titers in most studies reported resulted in a decreased attention of the potential blocking capacity of this IgG subclass [reviewed in ref. 23]. Recent reports from the field of parasitology, however, clearly suggested that IgG4 antibodies can indeed act as blocking antibodies, both in vivo and in vitro [25–29]. Husain et al. [29] demonstrated that IgG4 antibodies inhibited histamine release from human basophils induced by filarial antigen. An inhibitory effect of IgG antibodies on basophil histamine release induced by inhalant allergens was reported by some investigators [30–37]. In most of these studies, allergen and blocking antibodies were allowed to preincubate prior to addition to the basophils. We were interested to see whether allergen-specific IgG antibodies could also act as blocking antibodies without preincubation, an approach that resembles the in vivo situation more closely. To investigate this, we performed histamine release inhibition studies with human basophils and the major grass pollen allergen *Lol p I*. As source of blocking antibodies we used polyclonal rabbit antisera. Apart from a rabbit antiserum against natural *Lol p I*, we also applied rabbit antiserum obtained after immunization with a synthetic C-terminal peptide of *Lol p I*. In this way it was investigated whether immunization with a synthetic B cell epitope has the potential to induce a blocking antibody response.

Materials and Methods

Allergen Extracts and Purified Allergens

Grass pollens (*Lolium perenne*, *Dactylis glomerata*, *Phleum pratense*, *Secale cereale*, and *Phragmites communis*) were obtained from Diephuis Laboratories (Groningen, The Netherlands). The pollens were extracted as described earlier [38]. Freeze-dried housedust mite extract and cat dander extract were obtained from ALK (Horsholm Denmark).

Purified *Lol p I* was obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, Md., USA), NIH reagents A611–901–185.

Synthetic Peptide

The production of the synthetic C-terminal peptide used in this study was described elsewhere [39]. The sequence of the peptide *Lol p I*(216–240) corresponds to the 25 C-terminal amino acids of *Lol p I*: EGGTKSEVEDVIPEGWKADTSYSAK [40].

Human Sera and Basophil Donors

Sera ($n = 20$) used in a radioallergosorbent test (RAST) with Sepharose-coupled *Lol p I*(216–240) to measure IgG4 directed against the *Lol p I* C terminus were selected from sera of grass pollen allergic patients that received immunotherapy. The presence of specific IgG4 against *Lol p I*, as judged by a binding assay with radiolabeled *Lol p I*, was used as selection criterion ($>10\%$ binding for 1:40 diluted serum). As negative controls six sera from cat-allergic patients receiving immunotherapy were used. They all had specific IgG4 against *Fel d I* ($>10\%$ binding of radiolabeled *Fel d I*).

For the histamine release assays 3 allergic laboratory workers volunteered to provide blood. They had a positive RAST for grass pollen (4+/5+) and suffered from allergic rhinitis during pollen season. All 3 had IgE antibodies directed to *Lol p I* ($\geq 3+$). In addition, they had a positive RAST ($\geq 2+$) for house dust mite and/or cat dander. The RAST was performed as described elsewhere [41]. RAST classes are divided according to the percentages radiolabeled antihuman IgE bound: negative (0–2%), 1+ (2–5), 2+ (5–10), 3+ (10–20), 4+ (20–40), and 5+ ($>40\%$).

Rabbit Antisera

Rabbit polyclonal anti-*Lol p I* was prepared by immunization with purified *Lol p I*, as previously described [38]. For the production of rabbit antisera directed against the C terminus of *Lol p I*, 3 rabbits were immunized with 100 μg of *Lol p I*(216–240) (free peptide, without carrier protein) in complete Freund’s adjuvant. At 4-week intervals the rabbits were boosted with 100 μg peptide in incomplete Freund’s adjuvant. After three boosters the antibody titers against *Lol p I* and its C-terminal peptide did not increase anymore.

Radiolabeling

Lol p I and *Lol p I*(216–240) were radiolabeled by the chloramine T method with carrier-free ^{125}I (Amersham, Amersham, UK); 20 μg of allergen or peptide was labeled with 1 mCi of ^{125}I . Unbound ^{125}I was separated from labeled allergen or peptide by means of gel filtration (Sephadex G-75 or G-25, respectively).

Radioimmunoassay (RIA)

Radiolabeled *Lol p I* and *Lol p I*(216–240) were used in a binding assay for detection of rabbit IgG against *Lol p I* and its C-terminal peptide, respectively. Antibodies were captured with protein A-Sepharose

(Pharmacia). Per test 0.5 mg Sepharose, 5 µl of antiserum, and 10,000–20,000 cpm ^{125}I -labeled allergen or peptide were incubated in a final volume of 350 µl (PBS-AT). Results were expressed as percentages of binding of total counts added.

Radioimmunoprecipitation Assay (RIPA)

Sepharose-bound allergen from the RIA was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography, for determination of its molecular weight.

Competitive RIA

In a competitive RIA, rabbit antiserum was preincubated for 2 h with serial dilutions of pollen extracts prior to addition of Sepharose and ^{125}I -*Lol p I* (216–240). For determination of K_A and antigen-binding capacity of rabbit IgG, the competitive RIA was adapted for Scatchard analysis. Dilutions of purified *Lol p I* (10^{-1} to 10^4 ng/test) and 30,000 cpm of ^{125}I -labeled *Lol p I* (200 pg/test) were mixed prior to addition of antiserum and protein A-Sepharose (2.5 mg/test). The final volume was 400 µl (PBS-AT). Of rabbit anti-*Lol p I* 0.25 µl/test was used, of anti-*Lol p I* (216–240) 10 µl/test. K_A values were obtained by determining the slope ($-K_A$) of Scatchard plots and the antigen-binding capacity by determining the intercept on the abscissa (capacity of 400 µl diluted serum).

Histamine Release Assay

Red blood cells were lysed by incubating 10 ml of blood with 40 ml of 155 mM NH_4Cl /10 mM KHCO_3 /0.1 mM EDTA (pH 7.2) for 5 min at 4°C. After centrifugation a second lysis was performed in the same buffer. The cells were washed once with 0.9% NaCl and then resuspended in 2.6 ml Hepes medium (20 mM Hepes/132 mM NaCl/6 mM KCl/1.2 mM K_2HPO_4 /1 mM MgSO_4 /1 µM CaCl_2 /5.5 mM glucose/0.5% human serum albumin, pH 7.2). To determine the allergen concentration needed for maximum histamine release, 250 µl of this cell suspension was incubated, gently shaking at 37°C for 45 min, with 50 µl of ten-fold dilutions of allergen in Hepes medium (0.01 ng/ml to 10 µg/ml *Lol p I*; 0.1 to 100,000 SQ/ml house dust mite extract or cat dander extract). Inhibition with rabbit antisera (50 µl of crude or 150 µl of semipurified antiserum) was performed at suboptimal allergen concentrations. When allergen and antibody were preincubated, this was done for 1 h at 37°C. Differences in final volumes were eliminated by addition of extra Hepes medium. The maximum incubation volume (inhibition with semipurified antiserum) was 450 µl. The histamine content in the supernatant of the samples was measured with an automated fluorometric method, essentially as described by Siraganian [42]. Histamine release was expressed as percentage of the total histamine content of 250 µl cell suspension, released by perchloric acid precipitation. Percentages spontaneous release (identical incubation without allergen) were subtracted.

Results

Characterization of Rabbit Anti-*Lol p I* (216–240)

Three rabbits were immunized with a synthetic peptide of the 25 C-terminal amino acids of *Lol p I*: *Lol p I* (216–240). All three resulting antisera (R38100, R38101, and R38102) were not only reactive with this synthetic C termi-

Table 1. Binding of radiolabeled *Lol p I* and radiolabeled *Lol p I* (216–240) to rabbit anti-*Lol p I* (216–240)

Antiserum	Day	<i>Lol p I</i> (216–240)	<i>Lol p I</i>
R38100	0	0.3	0.4
R38100	117	53.8	34.5
R38101	0	0.9	1.1
R38101	117	38.6	21.4
R38102	0	0.4	0.7
R38102	117	75.3	33.0

Three rabbits were immunized with *Lol p I* (216–240), R38100/101/102, and tested (5 µl/test) for reactivity with the peptide and native *Lol p I* at day 0 and day 117 (after three boosters). Results are expressed as percentage binding of total counts added.

nus, but also with natural *Lol p I*. This was demonstrated in a binding assay with ^{125}I -*Lol p I* (216–240) and ^{125}I -*Lol p I* (table 1). Reactivity with *Lol p I* was confirmed by immunoblot (fig. 1A) and by a RIPA with ^{125}I -*Lol p I* (fig. 1B). Scatchard analysis revealed that anti-*Lol p I* (216–240) (R38102) had a 100-fold lower avidity for *Lol p I* than anti-natural *Lol p I*, K_A being $3 \times 10^7 \text{ M}^{-1}$ and $1.1 \times 10^9 \text{ M}^{-1}$, respectively. On the other hand, extrapolation of the Scatchard plots showed that both hyperimmune antisera had similar *Lol p I* binding capacity: 562 µg/ml and 254 µl/ml, corresponding to a calculated antibody content of 1.4 mg/ml and 635 µg/ml, respectively. Binding of ^{125}I -*Lol p I* (216–240) to R38102 was not only inhibited with *L. perenne* extract, but also with extracts of *D. glomerata*, *P. pratense*, *S. cereale*, and *P. communis*, demonstrating cross-reactivity with a large variety of grass species (not shown).

Inhibition of Basophil Histamine Release

Three subjects with a positive RAST for grass pollen were selected for histamine release assays. All 3 had specific IgE against *Lol p I* (>10% binding), but no detectable IgE against *Lol p I* (216–240) (<1% binding). For each of the patients used in this study the *Lol p I* concentrations needed for maximum histamine release from basophils (in washed leukocytes) were determined. For donors R.R. and H.G., the optimal concentration was 1 ng/ml, giving 33.4 and 57.9% histamine release, respectively. For donor H.W. the maximum release (61.2%) was achieved with 10 ng/ml. At higher concentrations less histamine was released.

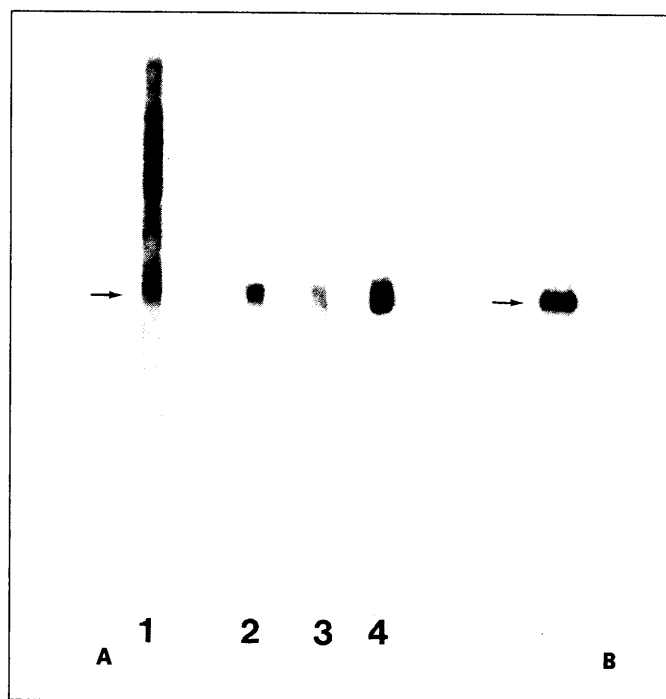


Fig. 1. A. Binding of rabbit anti-*Lol p I*(216–240) to *Lol p I* on immunoblot (after SDS-PAGE separation of crude *L. perenne* pollen extract). Lane 1 shows the binding of rabbit anti-*Lol p I*. The binding to high molecular weight bands above the *Lol p I* level is caused by cross-reactivity towards carbohydrate determinants. Lanes 2–4 show the binding of anti-*Lol p I*(216–240) (R38100, R38101, and R38102, respectively). The *Lol p I* band is marked with an arrow. **B.** SDS-PAGE/autoradiography of immunoprecipitated *Lol p I* (RIPA). Immunoprecipitation of radiolabeled *Lol p I* was performed with rabbit (R38102) anti-*Lol p I*(216–240).

Inhibition of histamine release by rabbit anti-*Lol p I* and rabbit anti-*Lol p I*(216–240) was studied at suboptimal *Lol p I* concentrations: 0.1 ng/ml for R.R. and H.G. and 1 ng/ml for H.W. (fig. 2). Inhibition was performed with and without preincubation of allergen and rabbit antisera. Preincubation of *Lol p I* and anti-*Lol p I* resulted in clear inhibition: 53.5–85.5%. Inhibition with anti-*Lol p I*(216–240) was less pronounced: 41.4–46.7%.

The typical decrease of histamine release at supraoptimal allergen concentrations was prevented by addition of rabbit antibodies against *Lol p I* or its C terminus. This is illustrated in figure 3 for donor R.R. with rabbit anti-*Lol p I* (216–240).

When allergen and antisera were not preincubated, only with anti-*Lol p I* inhibition was observed: 15.5–48.5% (fig. 2). Addition of antiserum did not significantly influence the histamine release with extract of house dust mite (shown for R.R. in figure 4), demonstrating the *Lol p I* spe-

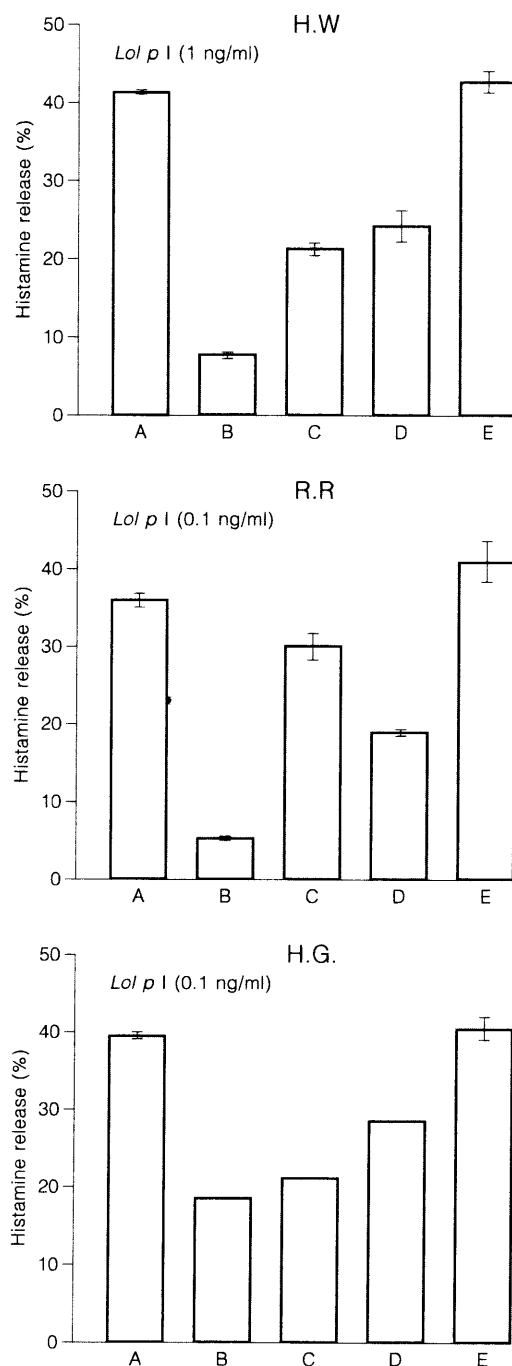


Fig. 2. Inhibition of *Lol p I* induced histamine release with rabbit IgG. Inhibition was performed with three donors (H.W., R.R., H.G.). A = Uninhibited; B and C = inhibited with anti-*Lol p I*; D and E = inhibited with anti-*Lol p I*(216–240) (R38102). Inhibition was performed both with (B and D) and without (C and E) preincubation of allergen and antiserum.

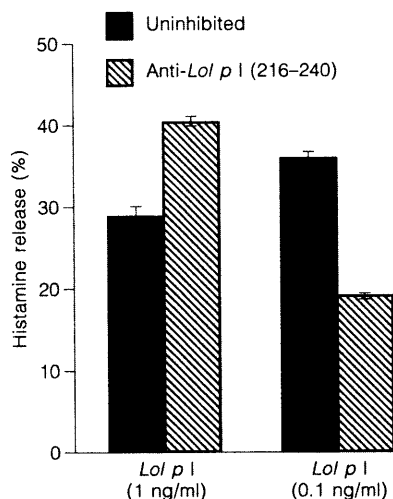


Fig. 3. Influence of rabbit anti-Lol p I (216-240) at supraoptimal Lol p I concentration. The effect is shown of preincubation of anti-Lol p I(216-240) with supraoptimal (1 ng/ml) and suboptimal (0.1 ng/ml) Lol p I concentrations on the histamine release from basophils of donor R.R. At supraoptimal allergen concentration, addition of rabbit antiserum resulted in an increased histamine release (28.6–40.4%); at suboptimal allergen concentrations, the histamine release was inhibited (35.9–19.1%).

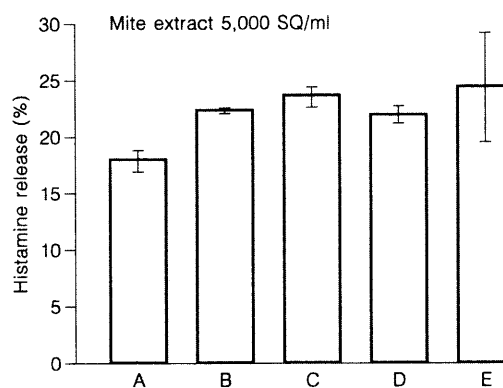


Fig. 4. Influence of antibodies on histamine release induced by house dust mite extract (donor R.R.). A = Uninhibited; B and C = inhibited with anti-Lol p I; D and E = inhibited with anti-Lol p I(216-240) (R38102). Inhibition was performed both with (B and D) and without (C and E) preincubation of allergen and antiserum.

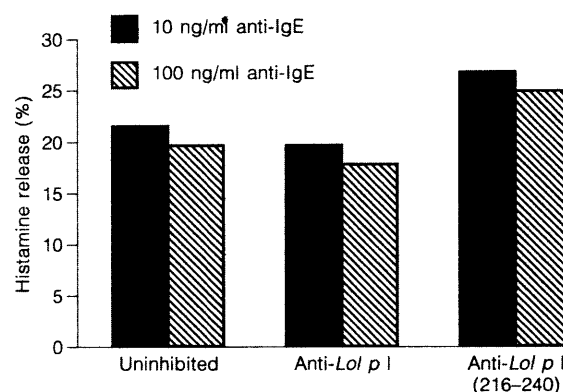


Fig. 5. Influence of rabbit antisera on anti-IgE-induced histamine release. Polyclonal sheep IgG against human IgE (SH25-1-p7; CLB) was used at two concentrations: 10 and 100 ng/ml. Both rabbit anti-Lol p I and anti-Lol p I(216-240) did not inhibit histamine release.

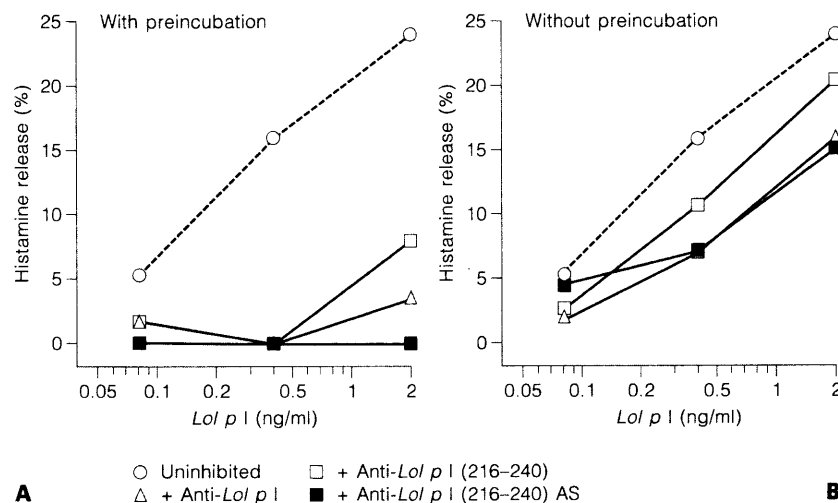
cific nature of the observed inhibition. Similarly, anti-IgE-induced histamine release was not inhibited by both rabbit antisera (fig. 5).

Inhibition with Partially Purified Rabbit Anti-Lol p I (216-240)

Besides its lower avidity for Lol p I, the lower inhibitory potency of anti-Lol p I(216-240) could also be caused by its limited epitope specificity. To find out whether the latter characteristic prevented complete inhibition, a larger quantity of anti-Lol p I activity isolated from the antipeptide antiserum was used. By means of ammonium sulphate precipitation, anti-Lol p I(216-240) was enriched for IgG, resulting in a three-fold increase in anti-Lol p I activity. By adding a three times larger volume than in case of the crude antiserum, inhibition was performed with almost ten times more anti-Lol p I activity. Three suboptimal Lol p I concentrations (2, 0.4, and 0.08 ng/ml) were used in histamine release inhibition assays with washed leukocytes of patient R.R. The difference in optimal allergen concentration with

the previous experiment (fig. 2) can be explained by the fact that both experiments were performed during two successive pollen seasons. Inhibition of histamine release was clearly increased by addition of more anti-Lol p I activity (fig. 6). Both with and without preincubation the inhibition observed with semipurified anti-Lol p I(216-240) was at least as good as with anti-Lol p I. When allergen and antibodies were allowed to preincubate, the semipurified antiserum resulted in >95% inhibition, indicating that for this

Fig. 6. Inhibition with rabbit (R38102) anti-*Lol p* I(216–240) after ammonium sulfate precipitation. *Lol p* I induced histamine release (donor R.R.) was inhibited with crude anti-*Lol p* I and with crude as well as semi-purified anti-*Lol p* I(216–240). **A** With Preincubation of allergen and antiserum (AS). **B** Without preincubation.



donor antibodies against one epitope can give complete inhibition. With crude anti-*Lol p* I(216–240) only 65% inhibition was reached at the highest *Lol p* I concentration. Inhibition was less pronounced without preincubation. Crude anti-*Lol p* I(216–240) resulted in 15 and 33% inhibition with 2 and 0.4 ng/ml *Lol p* I, respectively. With semipurified anti-*Lol p* I(216–240), the inhibition at these *Lol p* I concentrations increased to a comparable level as observed for anti-*Lol p* I (35 and 55%, respectively). Histamine release at a suboptimal cat dander concentration (100 SQ/ml) was not inhibited by addition of antibodies against *Lol p* I or against its C terminus (not shown).

Discussion

The most striking serological change during immunotherapy with allergen extracts is the induction of allergen-specific IgG4 antibodies [43]. These antibodies are classically referred to as 'blocking' antibodies, pointing at their possible role in capturing allergen before it can trigger effector cells, like mast cells and basophils. Many investigators have attempted to confirm this 'blocking' mechanism by monitoring induction of specific IgG4 antibodies and clinical efficacy. In a majority of these studies, no correlation of IgG4 titers with the clinical outcome of the therapy was demonstrated [23]. A central role of 'blocking' antibodies in the beneficial effect of immunotherapy was concluded to be unlikely. On the other hand, several observations point towards a role of IgG4 as 'blocking' antibodies.

High-specific anti-*Schistosoma haematobium* IgE titers are linked to protection against reinfection with these parasites [26]. It was demonstrated that patients with high-specific IgG4 titers are more sensitive to reinfection, pointing at a possible blocking effect of IgG4. A blocking effect of anti-helminth IgG4 was confirmed in vitro by inhibition of filarial allergen induced histamine release [29]. Recent studies from our laboratory suggested a similar role for IgG against inhalant allergens. When skin tests were performed with purified major cat allergen *Fel d* I, early-phase skin reactivity was significantly lower in patients with specific IgG against *Fel d* I [Witte, personal commun.].

An inhibitory effect of IgG antibodies on basophil histamine release induced by inhalant allergens was demonstrated in vitro by several investigators [30–37]. The main criticism on most of these studies is that inhibition was demonstrated after allergen and 'blocking' antibodies were preincubated, in some cases up to 1 h. This can hardly be regarded as a reflection of the way in which an allergen encounters 'blocking' antibodies in vivo. We wanted to investigate whether allergen-specific IgG antibodies are capable of inhibiting basophil histamine release without preincubation. Under these conditions, the presence of rabbit anti-*Lol p* I resulted in 15–55% inhibition of *Lol p* I induced histamine release, demonstrating that IgG antibodies can indeed act as 'blocking' antibodies without preincubation.

An allergen has to contain at least two IgE-binding determinants in order to be able to cross-link mast cell or basophil bound IgE. It was further demonstrated that histamine release was much more efficient with IgE tri- or te-

tramers than with dimers [44, 45]. For significant inhibition of histamine release with IgG antibodies, it is, therefore, not necessary to get blockade of all epitopes. Nevertheless, application of synthetic B cell epitopes in immunotherapy might become unpracticable if the number of different non-overlapping IgE epitopes, for 1 patient as well as for different patients, is too large. The number of epitopes is of course limited by sterical factors. At least for the 3 patients in this study, the number of epitopes on *Lol p* I, involved in cross-linking of basophil-bound IgE, seems to be limited indeed. This was illustrated by the finding that approximately 50% inhibition was achieved with an antiserum (R38102) obtained after immunization with only one synthetic peptide, *Lol p* I(216–240). This antiserum had a similar *Lol p* I binding capacity as antinatural *Lol p* I, but a 100-fold lower avidity for the allergen. Despite this, the antiserum was only ten times less potent as inhibitor of *Lol p* I induced histamine release than the anti-*Lol p* I antiserum. With an amount of semipurified anti-*Lol p* I(216–240) containing ten times more anti-*Lol p* I activity than the crude antiserum, >90% inhibition was observed. These results indicate that the lower inhibitory potency of the crude antiserum as compared with antinatural *Lol p* I was caused by its lower avidity and not by its limited epitope specificity.

Interestingly, none of the donors had specific IgE-recognizing peptide *Lol p* I(216–240). In contrast to the rabbit antibodies, their IgE possibly binds to the C terminus of *Lol p* I only in its native conformation. For safe application of a synthetic peptide in immunotherapy, it is of course an advantage when IgE does not bind. The nonallergenic proper-

ty of *Lol p* I(216–240) for the patients in this study was confirmed by the fact that the peptide did not cause histamine release from basophils (not shown). From an earlier study [39], however, we know that some sera contain specific IgE that binds to *Lol p* I(216–240). Whether histamine release from basophils of such patients can be induced by the peptide still has to be investigated.

It was demonstrated in a model system with rabbit antisera that blockade of allergen-induced histamine release is possible under conditions that closely mimic an in vivo situation, namely without preincubation of allergen and antiserum. Antibodies directed against a single synthetic epitope of *Lol p* I had the capacity to block histamine release induced by the natural allergen. Immunotherapy with grass pollen extract has been earlier shown to induce IgG antibodies reactive with this synthetic C terminus of *Lol p* I [39], indicating that this structure is antigenic in humans. If 'blocking' antibodies would be rehabilitated as part of the explanation how immunotherapy exerts its beneficial effect, peptides like *Lol p* I(216–240) could prove a safe alternative for natural allergens in inducing a blocking antibody response, although an increase in anti-*Lol p* I avidity would be necessary.

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