

Detection of IgE-binding activity in serum after intranasal treatment of normal rabbits with *P. judaica* extract

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Local intranasal immunotherapy relieves the allergic symptoms of rhinitic patients but little is known about the absorption and distribution of inhaled allergens in the body. The aim of this study was to establish whether allergenic proteins are able to reach the bloodstream by penetrating through the nasal mucosa when aqueous *P. judaica* extract was administered into the nostrils of normal rabbits. Optimal conditions for a sensitive modified RAST (immunocapture RAST) were set up and the method was used to detect clinically relevant allergenic activity in the systemic circulation. The kinetic profile after intranasal treatment was compared with the profile after intravenous injection of the allergenic extract. The findings are discussed in relation to the mechanism by which local immunotherapy acts.

Key words: allergenic extract; circulating allergen; immunocapture RAST; intranasal administration.

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Immunotherapy by subcutaneous injection of allergenic extracts has been used for decades in patients with allergic symptoms (22, 24). The mechanism underlying the clinical benefits of such treatment is not completely defined, but there is evidence that the induction of specific blocking antibodies, which confer immunological protection to patients, may greatly contribute (5, 17, 23).

However, the risk of dangerous side effects associated with the injection of allergenic extracts has led allergists to explore safer and more effective routes of administration.

Local intranasal hyposensitization has been recently proposed as an alternative to the traditional treatment (15, 19, 21, 30, 33). Intranasal administration by a proper inhaler of freeze-dried allergenic extract in lactose in-

duces a significant improvement of allergic rhinitis without side effects (2).

The mechanism of action of this treatment is still a matter of debate (6, 12, 19, 21). Proteins of the allergenic extracts may penetrate the nasal mucosa and reach the bloodstream. Although the nasal mucosa is thought to be one of the more important routes of allergic sensitization in man, little is known about the pharmacokinetic profile of absorption of inhaled allergens.

As we believe that the study of this kinetic pattern may cast useful light on the mechanism by which allergenic extracts act, we set up an *in vitro* method, called "immunocapture RAST", for the detection of IgE binding activity in serum after intravenous or intranasal administration of allergenic extract to normal

rabbits. This method has already been used by Urbanek et al. (32) to measure allergen-specific antibodies.

The present report covers our observations with *Parietaria* pollen extract, one of the main causes of allergic respiratory diseases in the Mediterranean area (7, 8, 28).

MATERIAL AND METHODS

Preparation of P. judaica pollen extract

Parietaria judaica pollen (*P. judaica*) was purchased from Allergon AB (Valinge, Sweden). Extraction was done (5% w/v) in 0.15 M phosphate buffer containing 0.1% sodium azide, pH 7.4 (PBS), for 24 h at 4°C under stirring. Particulate material was removed by centrifugation (18,000 rpm for 40 min); the supernatant was dialysed against distilled water in Visking tubes (Spectra/Por®, Oscar Mires, Milan, Italy) with nominal cut-off of 3,500 daltons, lyophilised and stored at -20°C.

Proteins were measured according to Lowry et al. (18) using bovine serum albumin as standard. The allergenic potency of *P. judaica* extract was determined by RAST-inhibition according to the procedure of Yman et al. (34) as regards the general principle, the FDA protocol (1) for the data plotting, and statistical validation criteria. Polystyrene beads were used as solid phase (Sferikit®, Laboratorio Farmaceutico Lofarma, Milan, Italy). Human sera were obtained by pooling sera from patients sensitive to *P. judaica* extract (RAST classes 3 and 4).

As test animals, New Zealand White rabbits (Charles River, Calco, Italy), weight 2.5–3.5 kg were used.

Treatment of animals

a) *Antisera production.* Antisera to *P. judaica* extract were raised in rabbits by subcutaneous injection of 1 ml of an emulsion of Freund's complete adjuvant containing 3 mg of protein

every 2 weeks for 2 months; then Freund's incomplete adjuvant was used for subsequent challenges done monthly for 10 months. One week after the last injection, blood was taken from the auricular artery and serial dilutions of the sera obtained were tested in immunodiffusion assay against the whole extract. Sera with a titer higher than 1/8 were pooled and stored at -20°C. When tested in crossed-immunoelectrophoresis this serum gave at least 10 main precipitin peaks (data not shown).

b) *Absorption study.* 100 µl of *P. judaica* extract, corresponding to 2.5 mg of protein, were instilled into each nostril of normal rabbits; 1 ml of physiological saline containing 1 mg of protein was injected into their auricular veins. For these experiments three animals per group were used.

Blood samples were obtained from the auricular artery at different intervals after administration of the extract and the corresponding sera were tested in immunocapture RAST.

Preparation of specific anti-P. judaica antibodies

The immunoglobulin fraction (mainly IgG) of sera from *P. judaica* extract-immunized rabbits was obtained by precipitation with ammonium sulphate (40% saturated solution), and the specific fraction was purified by affinity chromatography. Briefly, 6.0 ml of *P. judaica* extract in PBS containing 40 mg of protein, were covalently coupled to 8 ml of properly activated support (Affi-Gel 10/15®, Bio-Rad Labs, Segrate, Milan, Italy), by continuous gentle stirring for 4h at room temperature. After blocking residual reactive sites by 1M ethanolamine-HCl pH 8.0, gel was transferred to a column and washed with coupling buffer until optical density at 280 nm was close to zero. The ammonium sulphate-precipitated IgG fraction from hyperimmune rabbit sera was applied to this immunoabsorbent. Specific anti-*P. judaica* antibodies were then eluted with 0.1 M glycine-HCl and 0.5 M NaCl, pH 2.5, immediately neutralized by Tris 0.2 M, dialysed against PBS and stored in aliquots at -20°C. Protein concentration was estimated

on the basis of absorbance at 280 nm, assuming $E\ 1\%/1\text{ cm} = 14.0$.

Immunocapture (IC) and direct RAST techniques

Polystyrene beads (6.4 mm diameter, Precision Plastic Balls, Chicago, Illinois) were coated by an original procedure (4) with different concentrations of affinity-purified anti-*P. judaica* antibodies or with 20 µg of normal rabbit IgG (immunocapture RAST). These beads were placed in contact with 100 µl of appropriate dilutions of *P. judaica* extract overnight.

For direct RAST, beads were directly coated with 100 µl of the same dilutions of *P. judaica* extract as used in IC RAST.

The beads were washed with PBS containing 0.15% of bovine serum albumin (PBS-BSA), then incubated with 100 µl of RAST positive serum pool for 3 h. Bound IgE was detected after further overnight incubation of the beads with 50 µl of ^{125}I -anti IgE (Sferikit®, Laboratorio Farmaceutico Lofarma, Milan, Italy). The residual radioactivity was measured in a gamma-scintillation counter and the data are expressed as percent of bound radioactivity.

IC-RAST for plotting reference curve and detecting allergens in systemic circulation

Beads coated with the optimal concentrations of anti-*P. judaica* extract were placed in contact

either with known amounts of *P. judaica* extract (reference curve) or with blood samples from rabbits treated intravenously or intranasally (detection of allergen in systemic circulation), then processed as described for the IC-RAST technique.

RESULTS

Sensitivity

Initially, we compared the sensitivity of the IC-RAST method with that of direct RAST. For the IC method we sensitized beads with different concentrations of affinity-purified antibodies to *P. judaica* extract before putting them in contact with various dilutions of the extract. In direct RAST, beads were directly sensitized with *P. judaica* extract at the same concentrations as for IC-RAST. The results shown in Table 1 are representative of two different experiments. In IC-RAST the percentage of bound radioactivity was significantly higher than by direct RAST, suggesting that the IC method is more sensitive.

To verify the specificity of the binding in the IC method, activated polystyrene beads were also sensitized with a solution of 20 µg/ml of IgG from normal rabbit serum before contact with the same concentration of *P. judaica* extract. Nonspecific binding was negligible, reaching only 3.5% as maximum (Table 1). The small difference in bound radioactivity

Table 1.
Immunocapture RAST compared with direct RAST

Polystyrene beads coated with	<i>P. judaica</i> extract dilutions					
	1:200	1:1000	1:5000	1:25000	1:125000	1:625000
Rabbit anti- <i>P. judaica</i> IgG 20 (µg/ml)	37.8±0.98*	38.6±0.49	24.0±0.2	13.0±0.9	7.0±1.13	3.9±1.13
5	37.6±0.21	37.6±1.76	20.8±0.69	11.3±0.8	5.2±0.56	3.15±0.49
1.25	30.3±0.98	23.8±1.69	9.3±0.56	6.7±0.42	2.5±0.21	2.25±0.35
Rabbit normal IgG 20 (µg/ml)	3.5±0.21	2.25±1.21	1.9±0.28	1.85±0.07	nd	nd
Direct RAST	29.5±1.27	28.4±1.06	14.1±0.91	4.8±0.14	2.2±0.21	1.5±0.28

*Results are expressed as percent of bound radioactivity (mean±standard deviation)

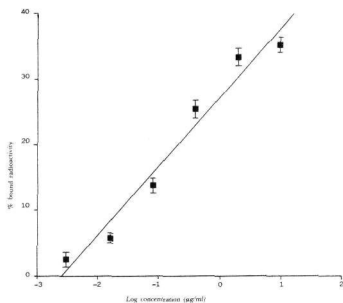


Fig. 1. Reference curve obtained using 5-fold dilutions of *P. judaica* extract in immunocapture RAST. Correlation coefficient was 0.98.

between 20 and 5 µg/ml of specific anti-*P. judaica* antibodies suggested that the system was close to saturation of the reactive sites. In all subsequent experiments, therefore, polystyrene beads sensitized with 10 µg/ml of affinity purified antibodies were used.

Detection of allergenic activity in sera of normal rabbits

In view of the good sensitivity of the IC method, we used it for detecting allergenic activity in sera after administration of *P. judaica* extract to normal rabbits by the two routes. We set up a reference curve adding known amounts of *P. judaica* extract, diluted in normal rabbit serum, to the anti-*P. judaica* antibodies sensitized beads, to simulate the *in vivo* situation as closely as possible.

A satisfactory dose-response curve was obtained in a wide range of concentrations of *P. judaica* extract (Fig. 1). We therefore administered the *P. judaica* extract to rabbits by intravenous injection (1 mg) and by the intranasal route (5 mg) and used IC-RAST to detect IgE binding activity in sera collected at different intervals.

The data in Table 2 are representative of three different experiments. Data about intranasal administration refers to undiluted sera, while intravenous treatment data refer to a 1:10 dilution of the sera as undiluted sera showed no time-response pattern because of an excess of IgE-binding activity.

From the data in Table 2, and using the reference curve of Fig. 1, the kinetic profiles shown in Fig. 2 were drawn. The maximum blood concentration (0.1 µg/ml) of IgE-binding activity was reached 60 min after intranasal administration of the extract. This was far lower than the concentration after intravenous administration when corrected for the differences in the dose, suggesting that there was partial absorption of allergenic activity through the nasal mucosa.

DISCUSSION

Passage through nasal mucosal tissues of soluble allergenic proteins from airborne material present in the environment is thought to be one of the mechanisms by which allergic sensitization is induced in humans. Indirect confirmation of this comes from the discovery of specific antibodies in sera after intranasal administration (3, 20, 29). On the other hand, such an antibody response could be the result of local sensitization that subsequently becomes systemic by recirculation of the lymphocytes involved from nasal mucosa to blood (11). However, there is no evidence of the presence of allergen in peripheral blood after administration of allergenic extracts or after natural exposure during the pollination period.

Kinetic studies based on measures of radioactivity remaining at the subcutaneous injection sites or found in other tissues of rats treated with ¹²⁵I-labelled allergenic extracts have been published (16); however, in our opinion such data are not completely probative because there was no immunological evaluation of the labelled material recovered. For example, the estimated radioactivity might have been due to protein components or fragments lacking allergenic properties. Our study using affinity-purified rabbit antibody-coated

beads was designed to detect the presence of true allergenic components in sera of *P. judaica* treated rabbits as recognized at the same time by IgE antibodies present in RAST-positive human sera.

Comparing the kinetic profiles obtained on intravenous and intranasal administration of allergenic extract (see Fig. 2), at the time of maximum absorption by the nasal route (60 min) the allergenic activity was about 10% of that detectable at the same time after intravenous injection. When corrected for the difference in the dose employed (1 mg, intravenous route; 5 mg, intranasal route) and determined by the weighing method of the area under the blood level-time curve (25), the bioavailability of IgE-binding activity after intranasal treatment was approximately 0.5% of that after intravenous injection.

This is not surprising considering that absorption through the nasal mucosa can be influenced by the molecular weight of a substance. Proteins from *P. judaica* extract have a molecular weight ranging from 10,000 to 80,000 daltons (9); allergen components with molecular weight > 10,000 daltons may encounter some difficulty in passing through the airway mucosa. It is likely that part of the administered extract was removed by nasal mucous clearance or by enzymatic degradation.

Detection of IgE-reactive components in the

systemic circulation when an allergenic extract is given intranasally introduces a new element of discussion about the possible mechanisms by which this form of treatment, recently proposed as an alternative to the parenteral route, produces clinical efficacy in rhinitic patients (15, 19, 21, 30, 33). Various hypotheses have been put forward: a common finding in allergic patients after local therapy is a rise in the threshold value as determined by specific provocation tests at the target organ, while the induction of local immunological protection (IgA and IgG blocking antibodies) is suggestive, but results are still contradictory (12, 15).

Recent studies in mice and rats showed that repeated inhalation of aerosols containing egg albumin or ragweed antigens induced a transient IgE response which switched off spontaneously despite continuous exposure (10, 13, 26). This seems to be mediated by activation of suppressor T cells in the lymph nodes draining the cavities of the nose and mouth (27). However, evidence is lacking that such a mechanism also operates in humans.

The clinical importance of our observations remains to be defined; certainly the amount of allergen absorbed by the nasal route is almost at the limit of the method's sensitivity, despite the high dose (2 mg/kg). In atopic patients the doses employed for immunotherapy are considerably lower. In this sense the amount absorbed is likely to be minute. However, our

Table 2.

Detection of IgE-binding activity in rabbit sera collected at different intervals after nasal (a) or intravenous (b) administration of *P. judaica* extract

a		b	
Time	% bound radioactivity (mean±SD)	Time	% bound radioactivity (mean±SD)
1'	0.3±0.1	1'	30.5±1.1
30'	12.0±0.3	30'	23.9±1.6
60'	16.5±1.1	60'	16.6±0.3
120'	9.2±1.5	120'	9.1±0.9
240'	5±0.9	240'	3.9±0.3
480'	1.2±0.4	480'	2.3
24h	nd	24h	0.9±0.2

a) 100 µl of *P. judaica* extract were instilled into each nostril, corresponding to a total of 5 mg of protein.

b) Rabbits were injected intravenously with 1 ml of saline containing 1 mg of *P. judaica* extract protein.

* Sera were collected immediately after the injection of *P. judaica* extract.

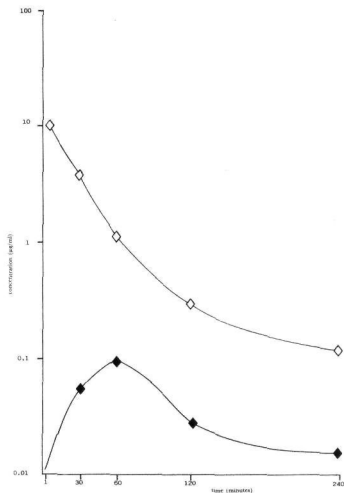


Fig. 2. Kinetic profiles obtained from reference curve referring to intranasal (●) or intravenous (◇) administration of *P. judaica* extract with 5 mg or 1 mg as protein, respectively.

studies were done on non-sensitized animals with an intact nasal mucosa; such a condition differs from that in clinical practice.

Firstly, there is evidence that in allergic patients the barrier function of the tight junctions of the nasal mucosa can be reduced, allowing the passage of macromolecular substances which usually cannot get through (14); this could enhance the penetration of more extensive allergenic activity, through the nasal mucosa of atopic patients reaching higher levels in the systemic circulation.

Secondly and contrary, specific antibodies in the mucosal tissue of the nose could take up allergen, limiting its transmucosal transport and amplifying its local effect. Soothill et al. suggested an etiological link between transient IgA deficiency in human infants and subsequent development of allergic disease (31).

In view of these contradictory observations, to understand the real situation better it would

be interesting to do a study in sensitized animals, provided a suitable experimental allergy model can be developed. Another area worthy of further experimental work concerns the use, as first antibody, of monoclonal antibodies against single allergens in order to establish which of them are capable of passing the mucosal barrier.

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