

Secretory IgA response in oral immunotherapy

Investigation in birch pollinosis

Taudorf E, Møller C, Russell MW. Secretory IgA response in oral immunotherapy. Investigation in birch pollinosis. Allergy 1994; 49: 760–765. © Munksgaard 1994.

A rationale for oral immunotherapy (OIT) might be founded on two potential mechanisms: induction of a mucosal secretory IgA response, or induction of systemic hyporesponsiveness (oral tolerance). Previous studies have shown clinically that there is a beneficial effect of OIT in birch pollinosis, in both children and adults. During OIT, birch pollen antigens in enterocoated capsules were given to 20 adults (participating in a double-blind, placebo-controlled trial) and 10 children, all suffering from birch pollinosis. Saliva and tears (only adults) samples were collected before, during, and after OIT. Each sample was assayed for both IgA antibodies against birch pollen antigens and total IgA by enzyme-linked immunosorbent assay. IgA antibody levels were also expressed in relation to total IgA concentrations, to correct for variations in secretion and flow rate between subjects and at different times. Changes in birch-specific secretory IgA antibodies in saliva and tears could not explain the beneficial effect of OIT in birch pollinosis. Further studies in this field are warranted.

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Key words: secretory IgA antibodies; oral immunotherapy; birch pollen; ELISA.

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Accepted for publication 24 February 1994

The mucous membranes are constantly exposed to numerous allergens and microorganisms, and the secretions bathing these membranes are thought to play a major role in their defense. Secretory IgA is quantitatively the most important immunoglobulin in mucous secretions (3). The secretory IgA immune response in mucous secretions has a well-defined role in limiting the passage of bacteria and viruses (10), and of potentially immunogenic macromolecules through the mucosal barrier. It has been shown that secretory IgA can act as a blocking antibody in nasal secretions and inhibit IgE-mediated histamine release (25). In this regard, it has been proposed that IgA deficiency, even if partial or transient, can be a predisposing factor in the development of allergy (4, 9, 18). Several studies of T-cell subsets in peripheral blood suggest that atopic children also have a decreased suppressor activity, a finding that might explain their enhanced IgE-producing capacity (7, 20). Sørensen & Kilian (21) found a significantly higher prevalence of bacterium-induced cleavage of IgA in the nasopharyngeal secretions from atopic children than in controls.

Recently, in double-blind, placebo-controlled tri-

als, a beneficial effect of oral immunotherapy (OIT) in birch pollinosis was shown in both children and adults (15, 22), although the underlying mechanisms for this beneficial effect have not yet been determined. One possible immunologic mechanism that could explain such an effect is induction of birch-specific IgA in secretions of the eye and nose via the common mucosal immune system (14), resulting in immune exclusion of birch pollen antigens at the mucosal surfaces (19).

This study investigated the levels of birch pollen-specific IgA antibodies in saliva and tears of allergic patients undergoing OIT.

Material and methods

Patients and treatment

Thirty patients, 20 adults and 10 children (13–16 years old) suffering from rhinoconjunctivitis from birch pollen allergy, entered the trial. Half of the patients in each group, i.e., 10 adults and five children, were actively treated with enterosoluble capsules containing a birch pollen allergen preparation, and the other half of the patients were given placebo

capsules. The capsules were administered daily to the adults with a top dose of 6×10^5 biologic units (BU), reaching a cumulative dose of 280×10^6 BU over 18 months. The children were treated for 7 consecutive days with a top dose of 1.4×10^6 BU every fourth week. One child stopped treatment 4 months after reaching a cumulative dose of 20×10^6 BU, while the other four children were given a cumulative dose of $50\text{--}100 \times 10^6$ BU in 10 months.

The patients comprised subgroups of those included in the studies by Taudorf et al. (22) and Møller et al. (15), where they are described in detail. Those participating in the present study were selected by a person who had no patient contact, and the other investigators remained blind as to their identity until the end of the study.

Ten nonatopic persons were included as normal controls.

Study design

In the adult patients, saliva and tears were collected at three different times, the first sample 1 month after discontinuation of a previous 1-year treatment (before the pollen season), the second sample 2–3 weeks after the treatment was started again (before the season), and the third sample at the end of the trial and in the middle of the birch pollen season.

In the children, saliva was collected at three different times, before treatment, after 3 months of OIT (just before the start of the birch pollen season), and after 10 months of OIT. No tears were collected in children.

In the nonatopic controls, samples were taken only twice, before and in the middle of the pollen season.

Sampling of saliva and tears

Whole saliva was collected in the morning, after the participants had been fasting and not smoking since midnight, in sterile plastic tubes and frozen at -80°C . Tears were also collected in the morning with the patient lying on a bed. No local eye medication was allowed for a day before tear collection. The lower eyelid was cautiously elevated, thus closing the lacrimal duct, and tear fluid accumulating at the lesser canthus was collected in 50- μl capillary tubes that had been blunted at one end by brief melting and cleaned in chromic acid. Approximately 100 μl tear fluid from each patient was then stored in plastic tubes at -80°C .

Saliva was collected from children in the middle of the morning. Salivation was stimulated with tablets containing fruit acids. Pure parotid saliva was collected from the right side with the aid of a special device. The samples were immediately frozen.

Assay of IgA antibodies in secretions

IgA antibodies against birch pollen antigens were assayed in saliva and tears samples by the enzyme-linked immunosorbent assay (ELISA). The antigen was Pharmalgen Birk t3, an extract of *Betula verrucosa* pollen antigens (Pharmacia/ALK Laboratorium A/S, Denmark). The highest strength lyophilized preparation (no. 4, equivalent to 100 000 BU/ml when diluted for injection as recommended) was used. Preliminary trials using sera known to contain antibodies against birch indicated that a concentration of 20 $\mu\text{g/ml}$ in 0.05 M borate-buffered 0.1 M NaCl, pH 8.4, was best, and Maxisorb Immuno-plates (Nunc, Roskilde, Denmark) were coated with the antigen by incubation with 0.1 ml per well of this solution overnight at room temperature. Control wells were left uncoated. Further preliminary trials indicated that assay of saliva and tear samples required different procedures for best results. For saliva samples, coated and control wells were blocked by filling with 10 mg/ml bovine serum albumin (BSA) in PBS (0.01 M phosphate-buffered 0.5 M NaCl, pH 7.2) containing 0.2 mg/ml NaN_3 , and samples were also diluted in the same solution. For tear samples, the blocking and diluent solution was 2.5 mg/ml gelatin in PBS-azide. Duplicate 0.1-ml samples of each of four serial twofold dilutions of saliva (diluted 1:50, 1:100, 1:200, 1:400) and tears (diluted 1:100, 1:200, 1:400, 1:800) were incubated in both antigen-coated and control wells overnight at room temperature.

After washing with four cycles of PBS containing 0.15% Tween 20, bound antibodies were revealed by treatment with 0.1 ml of horseradish peroxidase-conjugated antihuman IgA (P216, Dakopatts A/S, Glostrup, Denmark) diluted 1:1000 in PBS-Tween 20. After further washing, 0.1 ml of substrate, *o*-phenylenediamine dihydrochloride (0.5 mg/ml) and 1 mM H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.0, was applied for 15 min. Color development was arrested by the addition of 0.15 ml of 1 M H_2SO_4 , and absorbance was measured at 492 nm in a Titertek Multiskan photometer. For each dilution of sample, the mean absorbance given by control wells was subtracted from the mean absorbance obtained in antigen-coated wells. This correction was deemed to be necessary, because samples varied widely in the nonspecific reactions developed in uncoated wells. Further controls consisted of wells (coated and uncoated) not exposed to sample, but treated with the developing reagents. These always gave minimal background color development (≤ 0.05).

Assay of total IgA in secretions

Simultaneously with the specific antibody assay described above, each sample was assayed for total IgA content, also by ELISA. For this purpose, wells were coated with antihuman IgA (A262, Dakopatts) diluted 1:1000 in borate-buffered NaCl, pH 8.4. The blocking and diluent solution for both saliva and tears was PBS-Tween 20 containing NaN₃. Duplicate samples of saliva (diluted 1:10 000, 1:20 000, 1:40 000, 1:80 000) and tears (diluted 1:20 000, 1:40 000, 1:80 000, 1:160 000) were incubated in the wells overnight. It was repeatedly observed that replicate samples at these dilutions applied to uncoated wells resulted in color reactions no greater than the background obtained with coated or uncoated wells incubated without samples; therefore, these controls were routinely omitted. The assay was calibrated by applying on each plate a duplicate series of serial twofold dilutions of standard human IgA, starting at 250 ng/ml, prepared by diluting Human Serum Calibrator (X908, Dakopatts) in PBS-tween-azide. The plates were developed with peroxidase-conjugated anti-IgA and substrate as described for the antibody assay.

Calculation of results

Calibration curves were obtained by plotting log mean absorbance (y) against log IgA concentration (x), which gave linear plots having slopes of approximately 0.9. The dilution curves of the unknown samples in both antibody and total IgA assays gave similar slopes, and therefore were amenable to interpolation on the calibration curves. Thus, total IgA concentrations in µg/ml were obtained as the mean

of values corrected for each sample dilution factor. IgA antibody levels were similarly obtained and expressed as equivalent to ng/ml IgA. IgA antibody levels were also expressed in relation to total IgA concentrations, to correct for variations in secretion and flow rate between subjects and at different times.

Results

Tables 1–3 show results of total IgA, IgA antibody against birch, and IgA antibody levels in relation to total IgA concentrations in saliva and tears. As shown, we did not find any significant difference between placebo- and actively treated patients (adults and children) for any of the above mentioned variables in any samples during the study. We also examined individual results to determine whether a subgroup of actively treated patients showed any significant increase in their secretory IgA antibody against birch during OIT. None were found to do so.

Tables 4 and 5 show results of total IgA, IgA antibody against birch, and IgA antibody levels in relation to total IgA concentrations in saliva and tears for a nonatopic control group. In the control group, there was no change in total IgA and IgA-antibody levels against birch, and this group did not differ significantly from the two other groups.

The Mann-Whitney rank sum test was used for the statistical analyses.

Missing samples

It was difficult to collect tears; in some persons, it was impossible. That is the main reason for the small number in Table 2. In January 1984, saliva from only 11 persons (five active and six placebo) was col-

Table 1. Total and birch-specific IgA in saliva of adult patients

	<i>n</i>	Total IgA (Ig) μg/ml			IgA antibirch (Ab) ng/ml			Ratio Ab/Ig ng/μg		
		Mean	Range	SD	Mean	Range	SD	Mean	Range	SD
Sample 1 (Jan. 1984)										
Placebo (P)	6	218	75–396	127	387	55–1158	418	1.46	0.73–3.54	1.06
Active (A)	5	387	245–670	175	430	164–871	300	1.35	0.39–3.56	1.29
Significance limits										
P vs A			NS			NS			NS	
Sample 2 (Feb. 1984)										
Placebo (P)	8	288	62–633	188	170	39–278	72	1.31	0.11–2.81	1.00
Active (A)	9	250	23–572	193	274	37–806	302	1.06	0.62–1.93	0.47
Significance limits										
P vs A			NS			NS			NS	
Sample 3 (May 1984)										
Placebo (P)	9	148	51–300	128	290	3–1307	401	1.60	0.11–3.24	1.05
Active (A)	10	172	38–310	132	260	37–373	211	1.53	0.97–2.35	0.56
Significance limits										
P vs A			NS			NS			NS	

Table 2. Total and birch-specific IgA in tears of adult patients

	n	Total IgA (Ig) µg/ml			IgA antibirch (Ab) ng/ml			Ratio Ab/Ig ng/µg		
		Mean	Range	SD	Mean	Range	SD	Mean	Range	SD
Sample 1 (Jan. 1984)										
Placebo (P)	5	676	249–907	284	97	3–265	100	0.14	0.005–0.32	0.12
Active (A)	4	392	215–621	171	78	3–228	102	0.16	0.01–0.37	0.15
Significance limits P vs A			NS			NS			NS	
Sample 2 (Feb. 1984)										
Placebo (P)	7	581	430–1001	270	191	34–907	318	0.48	0.05–2.65	0.96
Active (A)	8	405	177–654	196	83	8–283	97	0.23	0.02–0.71	0.26
Significance limits P vs A			NS			NS			NS	
Sample 3 (May 1984)										
Placebo (P)	6	442	250–930	258	182	29–491	212	0.51	0.05–1.67	0.66
Active (A)	5	278	115–411	111	74	9–198	78	0.24	0.04–0.61	0.23
Significance limits P vs A			NS			NS			NS	

lected. Nine persons did not turn up for the test. In February and May 1984, only three active subjects and one placebo were missing. The same explanation applied to the nonatopic control group (Tables 4 and 5).

Discussion

A rationale for attempting oral hyposensitization might be founded on two potential mechanisms: induction of a mucosal secretory IgA antibody response, or induction of systemic hyporesponsiveness (oral tolerance). The observation that both may be induced simultaneously in experimental animals

(6) means that they need not be mutually exclusive. Orally induced tolerance has been described in numerous animal studies (24) using various agents. In most of these studies, tolerance was induced by enteric administration of antigen to unprimed animals. From a clinical point of view, this is of limited interest, since allergic subjects already have an ongoing IgE antibody production. However, Bloch et al. (1) were able to induce tolerance in previously primed rats by enteric immunization, and Lafont et al. (11) showed that antigen feeding in previously primed mice can interrupt reaginic antibody production.

The sensitivity of the patient as measured by

Table 3. Total and birch-specific IgA in saliva of child patients

	n	Total IgA (Ig) µg/ml			IgA antibirch (Ab) ng/ml			Ratio Ab/Ig ng/µg		
		Mean	Range	SD	Mean	Range	SD	Mean	Range	SD
Sample 1 (Dec. 1982)										
Placebo (P)	5	42.2	9–84	31.6	34.4	8–58	23.4	0.94	0.44–1.44	0.41
Active (A)	5	34.6	19–42	8.4	16.0	0–37	14.0	0.41	0–0.90	0.35
Significance limits P vs A			NS			NS			NS	
Sample 2 (Feb. 1984)										
Placebo (P)	5	78.4	10–186	66.3	55.8	16–126	42.4	0.90	0.52–1.60	0.46
Active (A)	5	33.6	16–56	12.8	16.6	0–47	16.5	0.39	0–0.84	0.30
Significance limits P vs A			NS			NS			NS	
Sample 3 (May 1984)										
Placebo (P)	4	32.0	10–63	21.5	24.3	5–43	13.7	0.96	0.36–2.10	0.67
Active (A)	5	32.0	11–73	21.9	22.0	4–62	21.5	0.57	0.22–0.85	0.22
Significance limits P vs A			NS			NS			NS	

Table 4. Total and birch-specific IgA in saliva of normal nonatopic adult controls

	<i>n</i>	Total IgA (Ig) μg/ml			IgA antibirch (Ab) ng/ml			Ratio Ab/Ig ng/μg		
		Mean	Range	SD	Mean	Range	SD	Mean	Range	SD
Sample 1 (Jan. 1984)	8	154	23–319	106	321	11–706	266	1.96	0.48–3.67	1.04
Sample 2 (May 1984)	10	196	22–543	150	289	54–1294	370	1.60	0.50–3.90	1.12

provocation tests is not directly related to levels of specific IgE (16). It has been suggested that there are two classes of IgE and that the clinical improvement of immunotherapy may partly be related to a conversion of IgE interacting with IgE, and not to IgE interacting with histamine-releasing factors (12). This could explain the decrease in the sensitivity of conjunctiva despite the increase of specific IgE during the first 3 months of OIT (15, 22, 23). The clinical improvement of immunotherapy in our studies may thus be due to a switch to more harmless IgE during the first part of the treatment. In the long run, there was a decrease of specific IgE, suggesting induction of tolerance. This does not exclude the possibility that formation of blocking antibodies on the mucosal surfaces is equally or even more important for the clinical outcome of OIT.

Turk et al. (25) measured nasal secretory antibodies in allergic persons (immunized and non-immunized) and normal subjects. All samples contained blocking antibodies as measured by their ability to inhibit antigen IgE-induced histamine release from human leukocytes. The antibodies were largely of the secretory IgA class. Platts-Mills et al. (17) measured IgA and IgG antibodies against ragweed pollen in nasal secretions. Their results did not support the hypothesis that allergic subjects are deficient in secretory IgA responses, but the study was not designed to investigate the secretory IgA response during immunotherapy and its clinical implications. Waldman & Bergmann (26) recently showed, in a very preliminary study involving only two volunteers, that the ingestion of 20 000 PNU of ragweed antigen in enteric-coated capsules over a period of 1 month resulted in a marked rise in nasal secretory IgA antibodies against ragweed. However, the clinical relevance of such a rise in nasal secretory IgA antibodies was not evaluated in ragweed pollinosis.

We were not able to detect a significant birch pollen IgA antibody response in secretions associated with the oral immunization regimen, or correlated with the observed clinical amelioration of sensitivity. However, before we conclude that the clinical improvement must be due to factors other than protective secretory IgA antibodies, certain additional factors must be considered. The considerable variations in flow rate of secretions such as tears and saliva between subjects and at different times adversely affect the concentration of immunoglobulins (2). However, this was compensated for in the present study, by simultaneous assay of both total IgA and antibody levels, so that specific antibody could be expressed as a proportion of the total IgA output. Storage of saliva and tears samples for approximately 4 years before assay may have resulted in some deterioration of the IgA antibody levels (5). This was not compensated for by assaying total as well as specific IgA antibody. At the time when the samples were collected, the sensitive IgA assay had not yet been developed. It was difficult to develop and that explains the delay between storage of the samples and the assay.

A mucosal IgA antibody response might be expected to operate by antigen exclusion (19). Protection from the effects of allergen challenge would then depend on the maintenance of an effective secretory IgA antibody barrier at the relevant mucosal surfaces. The common mucosal immune system allows the dissemination of an IgA response throughout all mucosal surfaces after antigenic stimulation, mainly through the inductive sites represented by the gut-associated lymphoid tissues (GALT) (14). According to this concept, general mucosal immunity may be monitored by assaying one of the more readily accessible secretions such as saliva or tears.

It must also be considered whether antibodies in

Table 5. Total and birch-specific IgA in tears of normal nonatopic adult controls

	<i>n</i>	Total IgA (Ig) μg/ml			IgA antibirch (Ab) ng/ml			Ratio Ab/Ig ng/μg		
		Mean	Range	SD	Mean	Range	SD	Mean	Range	SD
Sample 1 (Jan. 1984)	7	422	169–1186	346	146	6–363	129	0.48	0.013–0.85	0.35
Sample 2 (Feb. 1984)	9	266	127–545	149	97	20–268	89	0.35	0.13–0.84	0.28

saliva adequately represent those in secretions covering respiratory surfaces. Although saliva has been used satisfactorily to monitor mucosal IgA antibody responses after oral immunization (14), mucosal immunity may be selectively expressed at different sites, possibly because of the local presence of antigen. Whereas antigen-sensitized IgA-committed B lymphocytes are distributed from the GALT throughout the body, the mechanism of their homing to the glandular submucosae is not understood. Relevant antigens present at these sites may enhance the local response, perhaps by stimulating further cell proliferation before terminal differentiation (8, 27). However, we analyzed also the response in tear secretion, which is relevant to the protection of ocular tissues. Although IgA antibodies in tears showed no correlation with those in saliva, possibly indicating the role of local factors such as antigen, there was no association with the clinical effect either. Finally, mucosal IgA responses may not be sustained for long periods in the absence of continued or repeated stimulation (13). However, it is unlikely that the sampling schedule used in this study would have failed to detect responses even if they were already in a declining phase.

This study indicates that the clinical beneficial effect of OIT in birch pollinosis is not due to an induction of birch-specific IgA antibodies in secretions. However, more studies in this field are warranted.

Acknowledgments

The pollen antigens and antibirch antibodies were kindly supplied by Allergologisk Laboratorium A/S, Denmark. We thank Mrs Bente Hansen for excellent technical assistance with the assays and Mrs Birgitte Skovsted for skillful technical assistance in sampling saliva and tears. This study was partly supported by United States Public Health Service grant AI-18745.

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