

Detection of *Chlorella*-specific IgE in mould-sensitized children

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The content of IgE, specific to the unicellular green alga *Chlorella* sp., was analysed in sera from 46 atopic children sensitized to moulds, using radioallergosorbent test (RAST), immunoblotting and crossed immunoelectrophoresis/crossed radioimmunoelectrophoresis (CIE/CRIE). *Chlorella*-specific IgE was found in 23/46 sera by RAST, in 28/41 sera by immunoblotting and in 6/30 sera by CIE/CRIE. The *Chlorella* components most frequently binding IgE as analysed by gradient gel electrophoresis and immunoblotting were of molecular weights of approximately 13, 17, 19, 26 and 49 kD. Twenty-nine precipitating antigens, including seven IgE-binding precipitates were detected by CIE/CRIE. The study shows that low concentrations of specific IgE are formed to the green alga *Chlorella* in sera from atopic individuals sensitized to moulds.

Key words: allergen, *Chlorella*, CIE/CRIE, IgE, immunoblotting, microalgae, RAST

Accepted for publication 19 April 1990

CLINICAL ASPECTS

Green algae have previously been found to cause sensitization, especially in mould-allergic individuals with multiple sensitization. This work confirms that one of the most prevalent green algae in Scandinavia, *Chlorella*, induces only low levels of specific IgE antibodies in such individuals. This indicates that sensitization to green algae is probably of little importance in areas with a dry climate, like northern Europe. However, algae may contribute to allergic symptoms in individuals with symptoms during the humid part of the year, the mould season.

Clinical sensitivity to airborne microalgae has previously been demonstrated in several studies (2, 4-6, 11, 16). The frequency of skin reactivity to algae varies geographically and probably depends on the choice of algal strain, extract concentration, patients and test method. The algal genus most extensively investigated is the unicellular green alga *Chlorella* (2, 4-6, 11, 16). This genus is distributed all over the world in all kinds of environments and is often found in soils and as an aerophyte (12).

In earlier studies, we have investigated the IgE-binding components in different *Chlorella*

extracts using sera from serum donors, containing *Chlorella*-specific IgE but without documented clinical sensitivity to *Chlorella* (14, 15). In a clinical investigation we found a higher incidence of skin sensitivity to *Chlorella* in a group of mould-sensitive children than in an unselected atopic group (16). The purpose of the present study was to investigate the prevalence of *Chlorella*-specific IgE in sera from mould-sensitive children, using radioallergosorbent test (RAST), immunoblotting and crossed immunoelectrophoresis (CIE)/crossed radioimmunoelectrophoresis (CRIE).

MATERIAL AND METHODS

Twenty-eight sera from mould allergic children were provided from the Pediatric Allergy Out-patient Clinics at the Hospitals in Åbo, Finland, and Uppsala and Linköping, Sweden. These children had participated in a previous study on immunotherapy (7, 10) and sera were obtained before the start of immunotherapy. In addition, 18 sera were used from mould-sensitive patients in Linköping who had previously been tested for skin sensitivity to *Chlorella* (16). Six of these 18 children showed sensitivity to *Chlorella* in skin prick tests. Thus, a total of 46 sera from 22 girls and 24 boys, 6-17 years old, mean age 12.1, were investigated. The content of total serum IgE in these ranged from 18-1820 kU/l (median 315 kU/l).

To establish a CIE/CRIE reference pattern, three selected *Chlorella* RAST positive sera were pooled (3.2 PRU/ml to *Chlorella*). These sera contained *Chlorella*-specific IgE antibodies to a large number of *Chlorella* extract components as detected by immunoblotting. They also contained high amounts of total IgE (720-8300 kU/l) and specific IgE-antibodies to common inhalant- and food allergens. The same pool was used in earlier studies of the composition of *Chlorella* extracts (14, 15).

The extract of *Chlorella homosphaera*, previously described (15) was used. In brief, freeze-dried *Chlorella* cells were ultrasonicated in 0.125 M NH_4HCO_3 , 1/20 (w/v), pH 7.8, 4°C, for 8 × 1 min with cooling intervals in between. After clarification, the preparation was freeze-dried as a crude extract. Part of it was partially purified by hollow fibre ultrafiltration (Amicon®) to remove molecules below 3 000 Da and then freeze-dried.

The crude *Chlorella* extract was used as antigen in CIE/CRIE, while the partially purified extract was used for coupling to RAST discs and immunoblotting.

Rabbit antiserum was prepared essentially according to Harboe & Ingild (9). Three rabbits were immunized with freeze-dried *C. homosphaera* cells, first dissolved in Freund's complete adjuvant and after the three first immunizations in Freund's incomplete adjuvant. All bleedings

were tested in CIE and bleedings with the highest antibody titre were pooled.

Analyses of specific IgE

RAST was performed according to the manufacturers' instructions (Phadebas RAST®, Pharmacia Diagnostics AB), using RAST discs coupled with *Chlorella* extract (5 mg dry weight/ml). The mean of duplicate tests was used and the results expressed in Phadebas RAST Units (PRU)/ml. The limit for positivity was set to 0.20 PRU/ml based upon a negative control serum spiked with myeloma IgE (2 000 kU/ml). The radioactive uptake for the control serum was < 0.1 PRU/ml, i.e. less than half of that corresponding to 0.2 PRU/ml. The mean value of the coefficient of variation for duplicate tests was 12%.

Sodium dodecyl sulphate gradient polyacrylamide gel electrophoresis (SDS-gPAGE) and immunoblotting was performed essentially as described by Bengtsson et al. (3). The algal extract (0.2 mg protein/cm gel) was separated by SDS-gPAGE. Electroblotting was performed in a Tris-glycine transfer buffer with 10% methanol on to 0.22 µm nitrocellulose membrane. Serum incubation (dilution 1:3) was performed overnight and tracer antibody incubation (^{125}I -anti-IgE; Pharmacia AB; 200 000 cpm/ml) for 4 h. The X-ray film was exposed for 72 h at -70°C, using an intensifying screen. A negative serum spiked with myeloma IgE (2 000 kU/ml) was used as negative control. The nitrocellulose strips were scanned with a LKB Ultrosan XL and the radiostaining intensity of the bands was divided into three classes; weak (hardly visible; absorbance 0.03-0.1), medium (visible; absorbance 0.1-1.0) and high (heavily stained; absorbance > 1.0).

CIE/CRIE was performed according to Wallenbeck et al. (17). In CIE, 10 µl *Chlorella* crude extract (100 mg dry weight/ml) was run in the first dimension electrophoresis. In the second dimension electrophoresis, 20 µl rabbit antiserum per square centimeter gel was used. The

dried CIE plates were stained with Coomassie Brilliant Blue (CBB). For establishment of a CIE/CRIE reference pattern, dried CIE plates were incubated with the *Chlorella* RAST positive serum pool (0.4 ml diluted to 7 ml). To study the pattern of individual IgE-binding, sera from the mould-sensitive children were incubated. The X-ray film was exposed at -70°C at different time intervals, in order to classify the intensity of the IgE-binding according to Aukrust & Aas (1). Phosphate buffer (0.1 M), a negative serum, and a negative serum spiked with IgE (2 000 kU/ml), were used as negative controls. A maximal exposure time of 7 days, corresponding to CRIE-class G was used in order to detect all possible allergens. This gave background radiostaining of two antigens (Ag 8/9 and 18) in all samples including controls. This binding was not considered specific when evaluating IgE-binding in the patient sera.

RESULTS

Twenty-three of the 46 sera (50 %) from mould-allergic children were positive in RAST, containing *Chlorella* specific IgE of 0.21–11.5 PRU/ml, median 1.3 PRU/ml. Of the 18 patients, who previously had been subjected to skin prick tests with the same *Chlorella* extract as used in this study (16), six showed skin-sensitivity and had *Chlorella*-specific IgE as analysed by RAST.

Forty-one of the 46 patient sera were analysed with immunoblotting after size separation of the *Chlorella* components by SDS-gPAGE. Eighteen of the 23 *Chlorella* RAST-positive sera and 10 of the 18 *Chlorella* RAST-negative sera, i.e. totally 68 %, showed visible radiostaining of at least one band (Table 1). *Chlorella*-specific IgE was detect-

ed in all sera from the *Chlorella*-skin sensitive children. The intensity of the radiostaining in immunoblotting, measured by densitometry, varied but most bands were weak (Fig. 1). Components of molecular weights of approximately 13, 17, 19, 26 and 49 kD were most frequently found to bind to serum IgE (Fig. 1).

Twenty-nine precipitating antigens were found by CIE/CRIE in the crude *Chlorella* extract (Fig. 2a, b). The CIE/CRIE reference pattern of *Chlorella* sp. is demonstrated in Fig. 2c, with arbitrarily numbered immunoprecipitates. Seven of these immunoprecipitates, i.e. Nos. 5, 6, 14, 19, 20, 21 and 24, showed specific IgE-binding when using the pool of three selected RAST-positive sera (Fig. 2b, c). Six of the IgE-binding components could not be seen in CIE after protein staining with CBB (dotted lines in Fig. 2c).

Six of the 30 individual patient sera analysed in CRIE revealed radiostaining, CRIE-class D or weaker, directed against the seven *Chlorella*

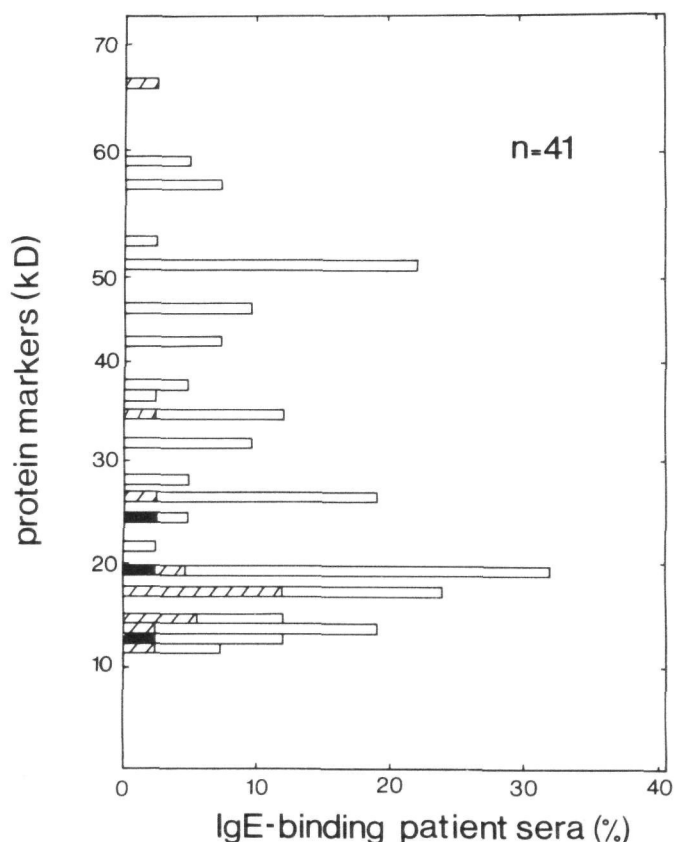


Fig. 1. Diagram showing IgE-binding to a partially purified *Chlorella* extract in 41 patient sera, detected by immunoblotting. The degree and frequency of radiostaining for bands of different molecular weights are shown; weak ($\square$), medium ($\square$) and heavy radiostaining ($\blacksquare$).

Table 1

IgE-binding to *Chlorella* extract components as analysed by RAST, immunoblotting and CIE/CRIE

Method	Total No. sera	RAST +	RAST-
Immunoblotting	41	23	18
IgE-binding detected	28	18	10
CIE/CRIE	30	20	10
IgE-binding detected	6	5	1

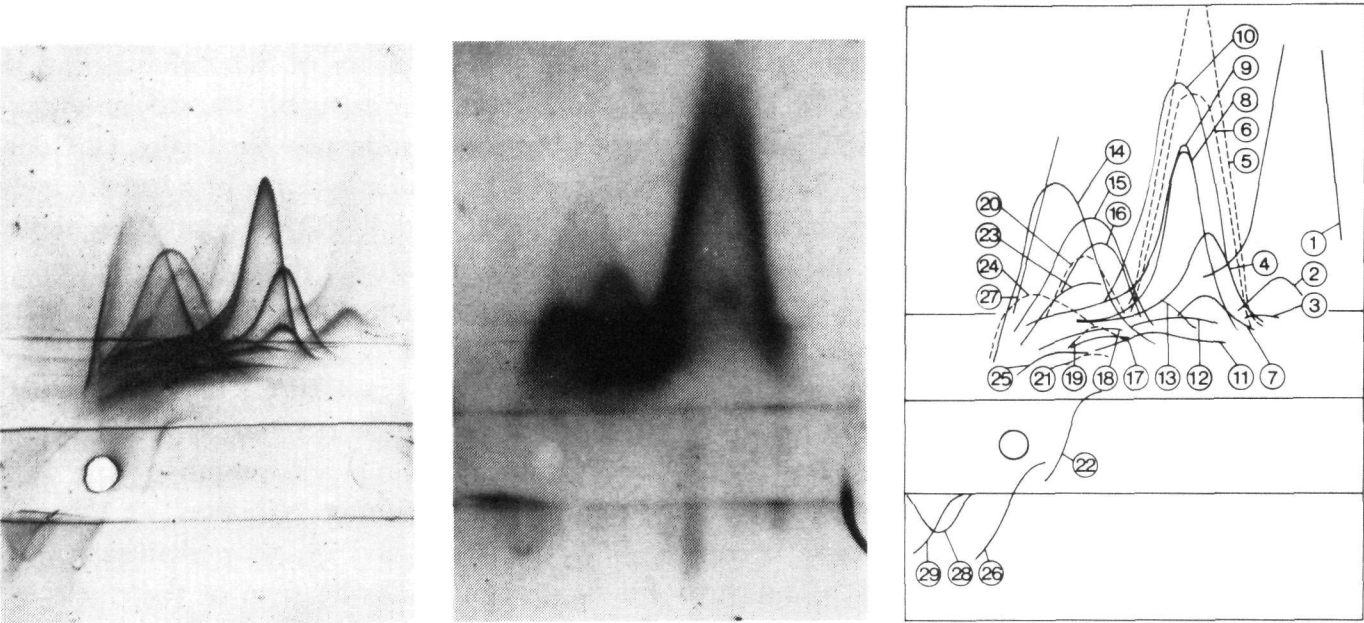


Fig. 2. CIE/CRIE reference pattern for *Chlorella* sp. Anode to the right in the first dimension and at top in second dimension electrophoresis. a) CIE stained with CBB. b) CRIE with a pool of three RAST positive sera. Exposure time 7 days. c) Schematic drawing of the immunoprecipitates detected in CIE and CRIE (reference pattern), arbitrarily numbered 1-29 from the anode. Precipitates seen in CIE (—) and precipitates only visible after radiostaining (----).

components detected with the reference serum pool (Tables 1, 2). One of the *Chlorella*-skin positive children showed IgE-binding to one *Chlorella* extract component in CIE/CRIE.

DISCUSSION

The finding of *Chlorella*-specific IgE by RAST in sera of 23 individuals of a panel of 46 mould-sensitive children is in accordance with our earlier findings of 30% *Chlorella*-positive individuals among mould-sensitive children by SPT and 40% positive by RAST (16). Further, by immunoblotting, 28 of 41 analysed sera revealed IgE binding to *Chlorella* components, and

in CIE/CRIE six of 30 tested sera showed specific IgE-binding (Table 1). In a previous study (15) no differences in the number of IgE-binding components and the intensity of binding was found by immunoblotting between the crude extract used for CIE/CRIE and the partially purified extract used for RAST and immunoblotting in this study. However, the allergenic potency of the purified extract, as measured by RAST inhibition, was increased compared with that of the crude extract due to removal of non-allergenic small compounds.

The RAST method is used to measure the total content of specific IgE antibodies. The sensitivity of the method depends on the allergenic components that have been coupled to the CNBr-activated cellulose paper discs. The lower sensitivity of *Chlorella* RAST compared with immunoblotting in this study was probably due to a lower coupling degree of allergenic proteins to the disc, compared with the transferred allergens to the nitrocellulose membrane.

In immunoblotting the proteins are immobilized on to nitrocellulose, while in CRIE, the proteins are immobilized by immunoprecipitation with rabbit IgG antibodies. Thus, the CIE/CRIE results depend on the quality of the rab-

Table 2

IgE-binding to *Chlorella* extract components analysed by CIE/CRIE in six patient sera

Patient	IgE-binding to antigen No.
1	24
2	14, 19
3	20, 21
4	6
5	5
6	6

bit antiserum, i.e. the presence of specific antibodies to the individual allergens. The *Chlorella* rabbit antiserum was prepared by immunization with whole cells, since whole cells cause a better stimulation of the immune response compared with that induced by proteins in solution (8). Antibodies were produced both to cell wall and intracellular antigens as localized in the transmission electron microscope by immunogold labelling of ultrathin *Chlorella* sections (data not shown). Even though CIE showed that the rabbits produced specific antibodies to a large number of *Chlorella* proteins (Fig. 2a) and specific IgE antibodies were detected to seven of them, rabbit IgG to certain IgE-binding components may have been of low titre, rendering the low sensitivity of CIE/CRIE in this study.

In RAST and CIE/CRIE a reference system based on serum from birch-sensitized patients was used, thereby ranking the amount of specific IgE in the analysed sera and enabling a comparison of the methods. However, in immunoblotting no such reference system was used. All radiostained bands which could be detected after 3 days' exposure of the X-ray film were considered to reveal specific IgE-binding to *Chlorella* components. The possibility of unspecificity was eliminated as a control serum with non-specific IgE did not show any radiostained bands. There was a relationship between the RAST and the immunoblotting results in that the sera with the highest RAST values showed the most pronounced radiostaining.

The molecular weight distribution of the *Chlorella* allergens, detected with sera from the mould-sensitive patients, was consistent with the distribution of *Chlorella homosphaera* allergens found when using sera from a serum bank, with positive *Chlorella* RAST, but without documentation of the clinical history of donors (14). The same serum pool was used for the construction of the CRIE reference pattern in this study (Fig. 2c) and the sera were selected for containing specific IgE to allergens over the whole molecular weight range as analysed by immunoblotting. This was probably the main reason for the detection of many IgE-binding components in CIE-CRIE when comparing this patient

serum pool with individual sera from the clinically sensitive patients.

Low levels of *Chlorella*-specific IgE were found by RAST, CIE/CRIE and immunoblotting. This may be due to low exposure of humans to microalgae, thereby causing minor sensitization. In Sweden, a maximum of 45 colony forming units (cfu)/m³ air was sampled in outdoor air, while the average was 3 cfu/m³ (13). Previously, sensitization was found among atopics with multiple sensitivities (16), i.e. individuals with genetic disposition to form IgE and with a possibly lowered exposure threshold for exhibiting allergic symptoms. It must be emphasized that the sera investigated in this study did not originate from clinically *Chlorella*-sensitive but possibly *Chlorella*-sensitive individuals. Therefore the demonstrated *Chlorella* allergens could not be classified into major, intermediate and minor allergens.

This study confirms that specific IgE is formed to the green algae *Chlorella* in atopic individuals, preferably those sensitized to moulds. Whether this is due to simultaneous sensitization or to cross-reactivity between moulds and *Chlorella* requires further investigation. *Chlorella*-specific IgE was present in levels high enough to be detected by immunochemical methods. However, the amounts of *Chlorella*-specific IgE in patient sera were low, confirming earlier indications that *Chlorella* does not cause sensitization of major importance.

ACKNOWLEDGEMENTS

We are indebted to Drs. A. Koivikko, Åbo, Finland, T. Foucard, Uppsala and N.-I. M. Kjellman, Linköping, Sweden, for providing us with sera from well-characterized patients. Ann-Marie Lindén is acknowledged for the production of rabbit antisera and Karin Yvell for skilful technical assistance. Katrin Österlund made the drawings. The Hierta-Retzius' and Samaritan Foundations and the Östergötland County Council gave financial support.

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