Allergy 1997: 52: 394–403
Printed in UK – all rights reserved

ALLERGY
ISSN 0105-4538

Anti-*Candida albicans* IgE and IgG subclasses in sera of patients with allergic bronchopulmonary aspergillosis (ABPA)

Roig E, Malo JL, Montplaisir S. Anti-*Candida albicans* IgE and IgG subclasses in sera of patients with allergic bronchopulmonary aspergillosis (ABPA).
Allergy 1997: 52: 394–403. © Munksgaard 1997.

E. Roig[1], J. L. Malo[2], S. Montplaisir[1]

[1]Department of Microbiology and Immunology, University of Montreal, Montreal, Quebec; [2]Department of Chest Medicine, Sacré-Cœur Hospital, Montreal, Quebec, Canada

We performed immunoblotting experiments to determine specific IgE and IgG subclass responses to *Candida albicans* antigens in allergic bronchopulmonary aspergillosis (ABPA) patients. This is a first report describing *C. albicans* antigens recognized by serum IgE and IgG subclasses of ABPA patients sensitized to that yeast. Among the various antigens reacting with serum IgE, a 43-kDa component was recognized by all seven patients and can be considered a major antigen of *C. albicans* for this particular group of patients. By comparison, only 20% of a group of asthmatic atopics (25 patients) and 10% of a group of normal controls (10 subjects) were 43-kDa positive. Multiple banding patterns, revealing no major antigen, were observed for all four IgG subclasses except for IgG1 in one case. In particular, the 43-kDa component was not always recognized by all the patients. Furthermore, oral or inhaled steroid treatment appears to have no impact on the specific IgE immunopatterns obtained. Using immunoelectron-microscopy, we localized IgE-binding primarily in the mannoprotein-containing layers of the *C. albicans* cell wall. In conclusion, *C. albicans*-IgE and IgG subclasses may participate in the physiopathology of ABPA by exacerbating pulmonary infiltrates (IgE) and inducing eosinophil-mediated inflammatory reaction (IgG1, IgG3).

Key words: allergen; allergic bronchopulmonary aspergillosis; *Aspergillus fumigatus; Candida albicans;* IgE; IgG subclasses; immunoblotting; skin prick test; cross-reactivity.

Dr S. Montplaisir
Department of Microbiology and Immunology
University of Montreal
C.P. 6128, Succ. Centre-ville
Montreal H3C 3J7
Quebec
Canada

Accepted for publication 12 November 1996

Allergic bronchopulmonary aspergillosis (ABPA) is a major pulmonary disease caused by the fungus *Aspergillus fumigatus*. The major diagnostic criteria include the presence of pulmonary radiographic infiltrates with peripheral eosinophilia almost always in asthmatics who show immediate skin reaction to *A. fumigatus*. Positive serum IgE and IgG (precipitins) to *A. fumigatus* antigens, elevated total serum IgE levels, and central bronchiectasis are also included among the primary criteria. The presence of plugs and *Aspergillus* hyphae in sputum and late skin reactivity to *A. fumigatus* are secondary criteria (1, 2). Frequently isolated from the environment, the fungal spores produce septate branching hyphae with characteristic swollen conidiophores. Among the antigenic components of *A. fumigatus*, a 20-kDa allergen has already been purified (3, 4).

ABPA patients have long been known to manifest hypersensitivity to *Candida albicans* antigens. Edge & Pepys have reported positive skin prick tests to antigenic extracts of *C. albicans* as well as specific serum IgE to that yeast among ABPA subjects (5). Many allergic manifestations, such as urticaria, asthma, and rhinitis, have been related to *C. albicans* (6–8). Studies by Savolainen et al. (9, 10) in children and young adults have shown a correlation between the severity of atopic dermatitis and IgE response to *C. albicans*.

C. albicans may play a role in the physiopathology of ABPA since this disease is often treated with corticosteroids, and such a therapy may constitute a predisposing factor for the pathologic growth of this yeast.

The objective of the present study was to determine the role played by anti-*Candida* IgE and IgG subclasses in the physiopathology of ABPA. Therefore, we collected serum samples from a group of patients attending a clinic for asthmatic patients. An immunoblotting analysis of *C. albicans*

IgE- as well as IgG subclasses-binding components was undertaken with sera from seven patients with ABPA, 25 asthmatic atopic patients, and 10 normal adults, with standardized antigenic extracts of both growth forms of *C. albicans*. An attempt to demonstrate cross-reactivity between antigens from *A. fumigatus* and *C. albicans* is also presented. In addition, an electron microscopy (EM) study was undertaken to determine the localization of IgE-binding components of *C. albicans* blastospores on thin sections.

Material and methods

Clinical data

Of 10 patients with ABPA followed at Sacré-Cœur Hospital in Montreal, seven agreed to participate in this study. Serum samples were obtained from those seven ABPA adult patients in chronic phase (three women and four men). The major diagnostic criteria in all cases were as follows: 1) asthma; 2) immediate *A. fumigatus* cutaneous reaction; 3) recurrent pulmonary radiologic infiltrates; 4) presence of peripheral eosinophilia at the time of infiltrate (1, 2). In addition, minor criteria such as elevated total IgE, *A. fumigatus*-specific IgE, and IgG (precipitins) were also present (Table 1). The patients were treated with oral or inhaled steroids (Table 1). Sera collected from adults belonging to a group of 25 asthmatic atopics (19 men, six women) and 10 normals (three men, seven women) without any history of allergy were selected as non-ABPA controls. Among the group of asthmatic atopics, all were positive for 5–11 tests out of a set of 15 skin prick tests including house dust, animal danders, grass and tree pollens, *Dermatophagoides farinae*, *Alternaria alternata*, *A. fumigatus*, and *Cladosporium herbarum*. Sera from three chronic mucocutaneous candidiasis (CMC) patients were also included.

Skin testing

Skin prick testing (SPT) was performed on three distinct sites on the forearm in order to obtain more reliable results with a panel of common inhaled allergens (Hollister-Stier and Bencard) including grass and tree pollens, animal dander, and fungi. The fungi included *A. alternata, C. herbarum, A. fumigatus,* and *C. albicans* (Hollister-Stier). Positivity was assessed by measuring the diameter of the wheal reaction (11), in comparison with a negative control, namely, sterile saline. The positive control was histamine-HCl (10 mg/ml).

Counterimmunoelectrophoresis (CIE)

Briefly, CIE was performed in agarose (50%) and Noble agar (50%) gel in sodium barbital buffer (pH 8.2), as described by Axelsen (12). *C. albicans* and *A. fumigatus* antigens were obtained from Sanofi-Pasteur (Montreal, Canada).

Total IgE measurements

A radioimmunoassay (RIA) was performed according to Sarfati et al. (13). Briefly, mouse monoclonal antibody (mAb) specific to human IgE (clone 89) was used at 10 µg/ml for coating on 96-well

Table 1. Treatment and laboratory findings in ABPA patients (1–7), asthmatic atopic patients, normal controls, and chronic mucocutaneous candidiasis (CMC) patients

	Steroid treatment		SPT reaction[c]			C.a.-IgE	CIE (ppt)[h]		C.a.-IgG subclasses[i] (immunoblot)			
Patients	Oral[a]	Inhaled[b]	C.a.[d]	A.f.[e]	Total IgE (kU/l)	AEU/ml[f] (class)[g]	C.a.	A.f.	1	2	3	4
1	None	400	–	+	590	0.4 (1)	neg	pos	–	+	++	+
2	None	2000	++	++	475	0.2 (0)	neg	pos	+	+++	++	+
3	10	2000	+	+++	11308	10.2 (3)	neg	pos	++	+	++	+++
4	20	400	+	+	974	0.2 (0)	neg	pos	+	+	++	+
5	10	2000	++	+	416	0.4 (1)	pos	pos	+++	++	++	++
6	None	2000	+	++	4970	0.2 (0)	neg	pos	++	+	+++	++
7	20	None	+	++	972	0.9 (2)	neg	pos	++	+++	++	+
Asthmatic atopics (*n*=25)	None	None	nt§	7/25#	566 ±313	0.09 ±0.07	7/25	5/25	nt	nt	nt	nt
Normals (*n*=10)	None	None	0/10	0/10	129 ±67	0	1/10	1/10	10/10	9/10	9/10	4/10
CMC (*n*=3)	None	None	nt	nt	88 ±23	0	pos	neg	2/3	3/3	3/3	1/3

[a] Prednisone: dose (mg) every 2 days; [b] beclomethasone: dose (µg) per day; [c] SPT: skin prick test, –: negative, +: 3 mm, ++: 4–5 mm, +++ >5 mm; [d] C.a.: *C. albicans*; [e] A.f.: *A. fumigatus*; [f] AEU/ml: Allercoat East Unit per ml; [g] (Class): 0–4 scale; [h] CIE (ppt): counterimmunoelectrophoresis (precipitins); neg: negative, pos: positive; [i] intensity of reaction from negative (–) to intense (+++); ‡ nt: not tested; # Positive number/total number.

polyvinyl microtiter plates (Dynatech Laboratories, Alexandria, VA, USA). After 2-h blocking with fetal calf serum supplemented with Hanks' solution and extensive washes, 75 µl Hanks' solution-diluted serum samples were incubated overnight at room temperature in a humid atmosphere. After several washing steps, the wells were reacted overnight with 75 µl of ^{125}I-labeled mAb to human IgE (3×10^5 CPM/75 µl). The wells were then washed, and radioactivity was counted in a gamma-counter. The optimal sensitivity of the assay was 30 pg/ml. Each serum was tested in duplicate by RIA.

Specific IgE measurements

Measurement of anti-*C. albicans* IgE was done by the enzyme-allergosorbent test (EAST, Kallestad, Chaska, MN, USA), according to the manufacturer's recommendations, using *C. albicans* allergen disks M5. The results were expressed in relation to a reference serum in Allercoat East Units (AEU/ml).

Antigenic extracts preparation

An *A. fumigatus* antigenic preparation was provided by Dr L. de Repentigny, Department of Microbiology and Immunology, University of Montreal, Montreal, Canada. Briefly, a fungus mat, obtained after 96-h incubation in liquid Czapek-Dox medium (17-l glass carboy) was homogenized with glass beads to produce a crude extract, as previously described (14).

C. albicans strain 4918 (serotype A), obtained from Dr R. Calderone (Georgetown University, Washington, DC, USA), was selected for the preparation of allergenic extracts of both growth forms. Washed yeast cells (2×10^8 cells/ml) were inoculated by spreading 100 µl onto synthetic medium agar plates, as described by Savolainen et al. (15) and grown for 48 h at 37°C. Sterile sodium chloride (0.9%) and collected yeast cells were centrifuged, and the pellet was frozen until extraction (adapted from 15, 16). For mycelium extract preparation, agar slants (2% glucose, 1% yeast extract, 2% agar) were inoculated with the same strain and incubated for 48 h at 25°C. Collected cells were grown in Lee et al.'s medium (17) for 5 h. Mycelial growth was then filtered and centrifuged, and the pellet frozen until extraction. *Saccharomyces cerevisiae* strain SEY 6210 (kindly provided by Dr P. Belhumeur, Department of Microbiology and Immunology, University of Montreal, Montreal, Canada) was grown under the same protocol as for yeast cells.

The cells suspended in sterile sodium chloride (10% w/v) were disrupted by shaking with 0.5-mm glass beads (50 g per 10 ml suspension) for 5 min (yeast form) or 8 min (hyphal form) in a Braun MSK cell homogenizer cooled with liquid CO_2. The slurry was separated from the beads with glass wool and centrifuged to 100 000 *g* for 1 h at 4°C. The clear supernatant under the turbid surface layer was collected and filter sterilized (0.22 µm) and then frozen, thawed, and centrifuged to eliminate any precipitate. The resulting supernatant was lyophilized.

The purified *C. albicans* yeast and mycelial extracts were the unbound material (carbohydrate-free) obtained after concanavalin A (ConA) Sepharose (Pharmacia) affinity chromatography at 4°C using 0.02 M Tris-0.5 M NaCl, pH 7.4, as loading and running buffer, according to Savolainen et al. (15).

SDS–PAGE and immunoblotting

SDS–PAGE (12% gel) of *C. albicans* (450 µg protein), *S. cerevisiae* (200 µg), or *A. fumigatus* extracts (75 µg protein) was performed with a Mini-Protean II apparatus (Bio-Rad) by a procedure described by Laemmli (18). Molecular weight (MW) was determined by comparison with molecular-mass standards (SDS-7, Sigma) included in each gel. Electrophoresis was run at 200 V constant voltage. Proteins were then transferred from the gel onto nitrocellulose membrane (Bio-Rad) with a semidry transfer cell (Transblot SD, Bio-Rad) and 25 mM Tris, 192 mM glycine, and 20% methanol buffer (19). Gels were stained with silver nitrate to control the efficiency of transfer, and standards and proteins transferred on membrane were revealed by the biotin-avidin-peroxidase technique. Polysaccharides (mannan and mannoproteins) were revealed after transfer on membrane by the ConA-peroxidase (50 µg/ml ConA or peroxidase in 50 mM Tris-HCl, 200 mM NaCl, 0.05% Tween 20, pH 7.4, 30-min incubation time for each solution) detection method with 4-chloro-1-naphthol (0.06% w/v) and hydrogen peroxide (0.01% in preceding buffer without Tween) (20). The membrane was cut into strips, which were incubated in phosphate-buffered saline (PBS)-0.05% Tween-1% nonfat dry milk (PTM) for 1 h at 37°C and then placed overnight in contact with individual serum. After three washing steps, strips were incubated for 5 h with murine ^{125}I-labeled mAb reacting to human IgE diluted in PTM (0.2 µCi/ml; 4×10^5 CPM/ml). The strips were thoroughly washed in PBS-Tween. After drying, the bands were placed on cardboard, wrapped with a plastic sheet, and exposed to radiographic film (Dupont Cronex 4) with intensifying screens in a cassette placed at –70°C for at least 1 week. IgG subclasses detection was performed similarly with

1 : 2500 diluted mAbs (Sigma, I9388, I5635, I7260, I7385) and ^{125}I-labeled antimouse Ig (0.2 μCi/ml, Dupont-NEN).

Ten umbilical cord-blood sera, obtained from the blood bank at Sainte-Justine Hospital (Montreal, Canada), were used as specific IgE negative control. Total IgE level was checked by RIA and specific IgE level by EAST.

Inhibition experiment

Serum adsorption was obtained after incubating overnight 250 μl patient serum with 250 μl *C. albicans* antigenic yeast form extract on a rotating wheel at 4°C. The extract was diluted in PBS and used at the following protein concentrations: 31, 15.5, 7.8, 3.9, 2, 1, 0.5, and 0.25 mg/ml. Sera were incubated with PBS as negative control. Bovine serum albumin (20 and 200 mg/ml) was used as control for nonspecific reactivity due to high protein concentrations.

Preparation of C. albicans *for electron microscopy and immunogold staining*

C. albicans 4454 M, serotype A, originally obtained from the Pasteur Institute, Paris, France, was obtained as previously described (21). After washing, culture was fixed with 3% glutaraldehyde (3 h) and 1% osmium tetroxide (3 h) in 0.1 M 1,4-piperazinediethane-sulfonic acid buffer (pH 7.4), at room temperature, with buffer rinses after each fixative. Cell pellets were infiltrated with 2% agar, dehydrated in a series of graded alcohols, taken into propylene oxide, and embedded in Araldite 502. Thin sections (80.0–100.0-nm-thick) were mounted on 400-mesh naked-nickel grids and processed for the immunocytochemical labeling, as previously described (21). Briefly, after the grids had been floated for 5 min on a drop of Tris-buffered saline (TBS) (0.05 M, pH 8.0) containing 0.05% Tween 20, they were transferred to diluted patient serum in TBS-Tween for 60 min at 22°C, washed in TBS-Tween, and incubated for 1 h at 22°C on a drop of mAb specific to human IgE (same as for immunoblotting), followed by washing steps and further incubation for 1 h at 22°C with goat antimouse IgG conjugated with 10-nm colloidal gold particles (Janssen Life Sciences Products, Olen, Belgium) and diluted 1 : 20 in TBS. After washing with TBS, rinsing in water, and drying, the sections were counterstained with uranyl acetate and lead citrate and then examined in a Philips EM 300 electron microscope. Specificity of the labeling was demonstrated by controls, including incubation with mAb specific to human IgE and gold-conjugated antimouse IgG, with colloidal gold-conjugated antibody alone, *C. albicans*-adsorbed patient serum, and umbilical cord-blood serum.

Results

SPT with common allergens, and with C. albicans *and* A. fumigatus *allergenic extracts*

Four out of seven ABPA subjects presented positive SPT for grass and tree pollens, cat and dog dander, and house dust, and two out of four tested were also positive for *A. alternata*. The 25 asthmatic atopic subjects reacted to various common allergens. In addition, in this last group, 12 were SPT positive for *A. alternata* and 10 for *C. herbarum*. All seven ABPA patients showed positive SPT to *A. fumigatus* allergenic extract. Among the 25 asthmatic atopic patients, seven were positive, and none of the 10 normal controls reacted (Table 1).

The *C. albicans* extract provided by Hollister-Stier was chosen for SPT in the seven ABPA patients, since a greater number of separate bands was obtained after running SDS–PAGE with this extract than the Bencard extract. Six out of seven ABPA patients presented a positive wheal reaction, and the 10 normal controls were all negative (Table 1).

C. albicans-*EAST determinations*

Anti-*C. albicans* IgE levels were determined by EAST. Specific IgE levels varied from undetectable (three patients), to low (two patients), medium (one patient), or high (one patient). The non-ABPA asthmatic atopic patients showed varying levels of specific IgE (EAST class 0–2). No detectable *C. albicans*-IgE level was obtained with 10 normals and three CMC subjects, and 10 cord-blood specimens (Table 1).

IgE-A. fumigatus *immunoblotting*

The seven ABPA subjects were tested for *A. fumigatus*-specific IgE (Fig. 1). All patient sera reacted with four major bands, ranging from 45 to 66 kDa at variable intensity. Antigens were weakly recognized by the serum of patients 1 and 2, in contrast to patient 3, who reacted very strongly to multiple antigens. An intermediate reactivity was observed for patients 4, 5, 6, and 7. A component of 23 kDa was recognized by only one serum (patient 6), whereas the 13- and 26-kDa bands were recognized by five and three patients, respectively. The umbilical cord-blood specimens were negative.

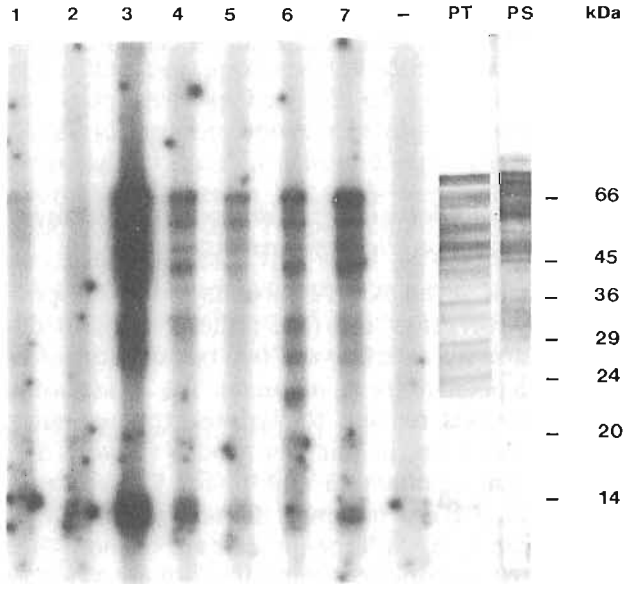

Fig. 1. Immunoblotting analysis of *A. fumigatus* IgE-binding components of ABPA patient sera (1–7). Proteins were separated by SDS–PAGE and transferred to nitrocellulose. Protein (PT, biotin-avidin-peroxidase staining) and polysaccharide (PS, ConA staining) profiles of allergenic extract and molecular weight standards are shown on right side of figure. Negative control: –.

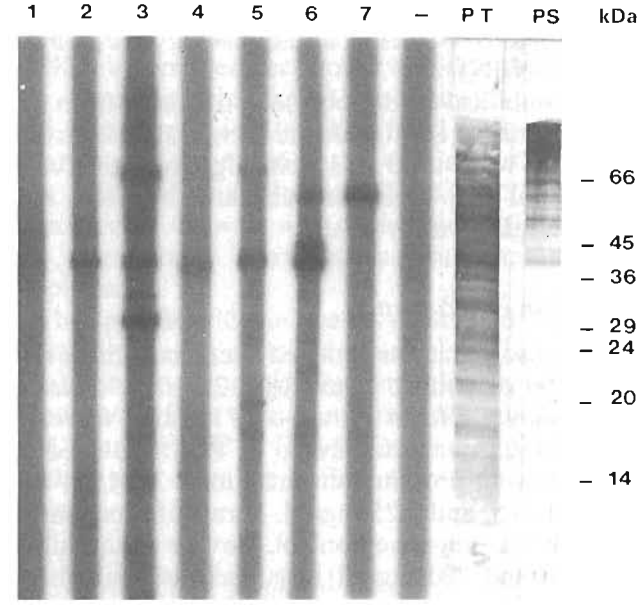

Fig. 2. Immunoblotting analysis of *C. albicans* IgE-binding components of ABPA patient sera (1–7). Protein (PT) and polysaccharide (PS) profiles of allergenic extract are shown on right side of figure with molecular weight standards. Negative control: –.

IgE-C. albicans *immunoblotting*

Anti-*C. albicans* IgE were detected in all the seven ABPA subjects, as shown (Fig. 2). Antigenic components presenting IgE binding were mainly localized in the range 18–66 kDa. It also appeared that a component having an estimated MW of 43 kDa extracted from yeast as well as mycelium was reactive for all the seven ABPA patients (Table 2). After ConA affinity chromatography, the reaction remained positive against both purified extracts (carbohydrate-free). In addition, asthmatic atopic patients presented reactive bands with yeast and mycelial extracts to a degree varying from 48 to 80% (Table 2). In this last group, the reactivity to the 43-kDa component was variable (8–28%). Although the serum of patient 1 had a negative SPT to *C. albicans*, it reacted to the 43-kDa component. The serum of patient 3 reacted strongest to the 43-kDa band as well as to six other components. In the zone known to be essentially constituted of mannan and mannoprotein (MW greater than 60 kDa), only two patient sera (nos. 3 and 5) reacted. Among the 25 asthmatic atopic patients, 15 (60%) were anti-*C. albicans* (yeast extract) IgE positive, in comparison to 2/10 normal controls (20%). The positive sera of these two last groups presented a variable pattern in number and intensity of bands, and no particular antigenic component was identified. Although the three CMC patients presented negative *C. albicans*-specific IgE, as determined by EAST, one of them presented a weak positive reaction to the 43-kDa component present in the yeast-purified extract (Table 2). Finally, we observed an increased number of positive reactions after using a *C. albicans* mycelial extract rather than a yeast extract (Table 2).

Comparative analysis of IgE reactivities to allergenic extracts of C. albicans, S. cerevisiae, *and* A. fumigatus

The two strongest *C. albicans*-IgE reacting sera, namely, the serum of patient 3 (Fig. 3, lane 2) of the ABPA group and one serum from the asthmatic atopic group (Fig. 3, lane 1), were retested by immunoblotting after adsorption with *C. albicans* yeast extract (2 mg/ml protein, in Fig. 3) in order to verify possible cross-reactivity with *A. fumigatus*. The adsorption was effective since the reactivity disappeared completely or partially when assayed against *C. albicans* yeast or mycelium form, respectively. However, in the heterologous reaction, the reactivity to *A. fumigatus* remained unchanged after adsorption, even with more diluted serum (result not shown), indicating that the anti-*C. albicans* IgE did not cross-react with *A. fumigatus*. Two additional ABPA patient sera (nos. 6 and 7) were also tested similarly (i.e., with the same range of *C. albicans* concentrations (0.25–31 mg/ml) for both homologous and heterologous reactions), and

Table 2. Detection of serum IgE by immunoblotting with crude and purified (carbohydrate-free) yeast and mycelial extracts, in ABPA patients (1–7), asthmatic atopic patients, normal controls, and chronic mucocutaneous candidiasis (CMC) patients

	Yeast extract				Mycelial extract			
	Crude		Purified		Crude		Purified	
	Bands	43 kDa	Bands	43 kDa	Bands	43 kDa	Bands	43 kDa
1	2§	+	4	+	2	+	4	+
2	1	+	1	+	1	+	1	+
3	7	+	9	+	6	+	6	+
4	4	+	5	+	2	+	4	+
5	10	+	9	+	7	+	10	+
6	10	+	9	+	3	+	5	+
7	8	+	11	+	4	+	6	+
Total	7/7§	7/7	7/7	7/7	7/7	7/7	7/7	7/7
Asthmatic atopics ($n=25$)	15/25 (60%)	5/25 (20%)	12/25 (48%)	6/25 (24%)	20/25 (80%)	2/25 (8%)	18/25 (72%)	7/25 (28%)
Normals ($n=10$)	2/10 (20%)	1/10 (10%)	1/10 (10%)	1/10 (10%)	nt†	nt	2/10 (20%)	2/10 (20%)
CMC ($n=3$)	0/3	0/3	1/3	1/3	nt	nt	nt	nt

§Number of bands; # positive number/total number; † nt: not tested.

showed no reduction in IgE reactivity to *A. fumigatus*. As an additional control, the two sera were tested before and after adsorption with the same *C. albicans* extract against *S. cerevisiae*. The results obtained demonstrated some cross-reactivity between these two fungi; in particular, a 32-kDa component (Fig. 3, Sc).

Immunolocalization of IgE-binding components on C. albicans *thin sections*

C. albicans thin sections were sequentially treated with patient sera and murine mAb specific to human IgE, followed by gold-labeled goat anti-mouse IgG. With sera of patients 2 and 3, labeling could be seen principally in the outermost and innermost layers of the blastospore cell wall (Fig. 4). Rare gold particles can be occasionally detected between those two layers and in the cytoplasm of the blastospores. As a negative control, thin sections were incubated with umbilical cord-blood serum, which gives only low background. A similar localization pattern of the gold staining was observed with all the patient sera. Using *A. fumigatus* thin sections, we were also able to demonstrate IgE binding on the cell walls of *A. fumigatus* conidia and hyphae, as already shown by Reijula et al. (22).

*IgG subclass-*C. albicans *immunoblotting*

Sera from ABPA patients contained antibodies belonging to the four IgG subclasses reacting to various *C. albicans* antigens, as detected by immunoblotting (Table 1). Individual sera presented reactions varying from 0 to 18 bands in the MW range 29–200 kDa. Only the serum of patient 1 did not contain IgG1 reacting to *C. albicans*, but the three other subclasses were present; the six remaining patients presented a pattern of IgG1 varying from 2 to 14 bands, serum number 3 being the only one reacting to the 43-kDa component; the three CMC patients presented a pattern of 9–12 bands, and only one reacted to the 43-kDa component. The seven ABPA patients presented an IgG2 immunopattern varying from 1 to 17 bands at variable intensity; four patients (nos. 2, 4, 5, and 7) presented a positive reaction to the 43-kDa component; the three CMC patients were IgG2 positive and displayed 2–6 bands, and none reacted to the 43-kDa component. The *C. albicans* IgG3 subclass was detected in the sera of all ABPA patients as well as of the three CMC patients, and they all reacted to the 43-kDa component except two (ABPA patient 2 and one CMC patient). Finally, the IgG4 immunopattern was positive for the seven ABPA patients and for only one CMC patient, and none reacted to the 43-kDa component. The IgG4 presented preferentially a diffuse signal in the high MW zone.

Discussion

In 1873, fungal hypersensitivity was described by Blackley (23), but still there are no adequate extracts with which to diagnose fungal allergy. In general, patients present sensitivity to many other allergens, making it difficult to determine the true influence of fungi on clinical symptoms.

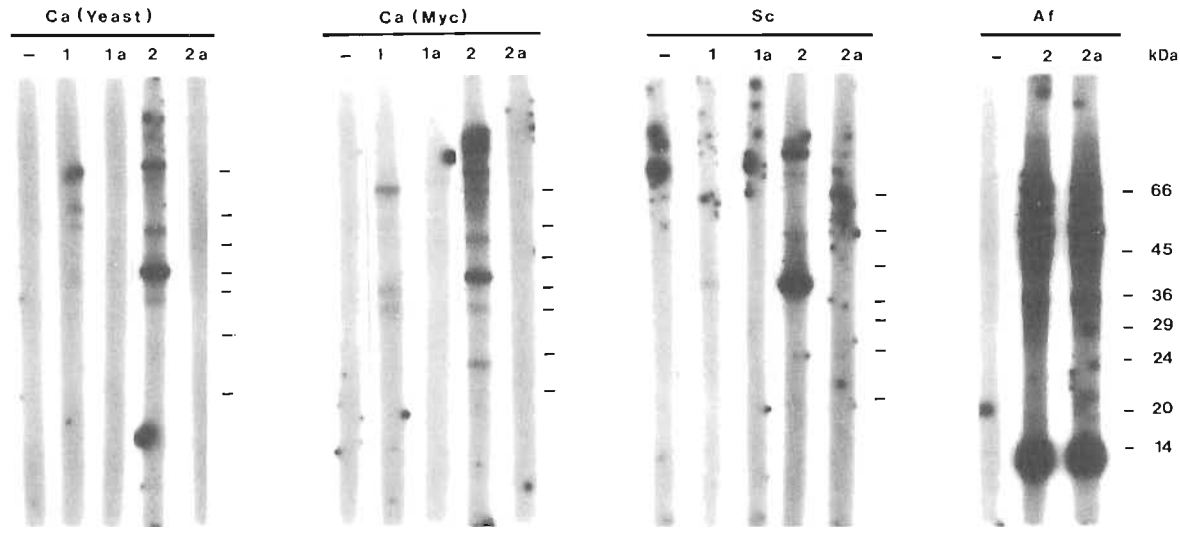

Fig. 3. Comparative immunoblotting analysis of *C. albicans* (Ca [yeast and mycelium]), *S. cerevisiae* (Sc), and *A. fumigatus* (Af) IgE-binding components of atopic patient's serum (lane 1) and serum of ABPA patient 3 (lane 2) before (1 and 2) and after adsorption (lanes 1a and 2a) with *C. albicans* extract. Molecular weight standards are shown on right side of each panel of figure. Negative control: –.

C. albicans has also been reported to participate in various ways in allergic disorders. In 1980, Edge & Pepys (5) analyzed antibody classes to *C. albicans* produced by patients with allergic respiratory diseases such as rhinitis, asthma, or ABPA and found *C. albicans*-specific IgE in 75% of the patients belonging to all three groups but without reference to any particular antigen. Studies conducted by Savolainen et al. (24, 25) have shown increased levels of IgE, IgA, and IgG antibodies against *C. albicans* among atopic and asthmatic patients. The exact role of the IgE response to that yeast among these patients has never been clearly established. In a clinical investigation conducted by Koivikko et al. (26), no association was found between the symptoms and occurrence of *C. albicans* or other yeasts, but immediate skin reactivity showed a positive correlation with the presence of *C. albicans*. Moreover, *C. albicans*-IgE response was associated with *C. albicans* nasopharyngeal growth, but not with the cell-mediated candidin skin test response, which was lowered (24).

One important task in clinical allergy is to identify allergenic components and define chemical composition in the commercially available crude extracts that are used to diagnose and treat allergic diseases. Immunoblotting is the method of choice, being largely used for identification and characterization of allergens and antigens in a crude extract. To our knowledge, such a characterization has not been undertaken in ABPA patients for *C. albicans* antigens.

We have tested the sera from seven ABPA patients for IgE reactivity specific to *A. fumigatus* allergens and showed positive reactions. Allergens detected in these cases slightly differ from those recently reported by Leser et al. (27) and may reflect antigenic differences between fungal extract composition due to preparation. We used a glass-bead-disrupted fungus mat, as opposed to the culture filtrate used in their study. Sera from the selected patients were all drawn during the chronic phase of the disease, and consequently all the distinct patterns observed refer to the same clinical phase. The weak reaction patterns observed for patients 1 and 2 may be due to the fact that the specimens were not collected during exacerbation of symptoms.

In the present study, we demonstrate a positive IgE binding to different antigenic components of *C. albicans* in all the seven ABPA patients tested. Each patient displays a distinctive IgE-binding pattern with variable intensity for each band. This is the first study demonstrating that a 43-kDa component, present in yeast as well as in mycelium extracts, reacts with IgE in sera of patients with ABPA. This component should be considered a major allergen of *C. albicans* among this particular group of patients. Reactivity to this 43-kDa component is retained in the purified extract (unbound material after ConA chromatography), suggesting that it is not composed of mannose or glucose residues with affinity for ConA. IgE binding to components of similar MW has already been described among other groups of atopic and asthmatic patients (24, 25). The chemical nature of these allergenic components needs to be elucidated since a precise evaluation of their MW remains difficult.

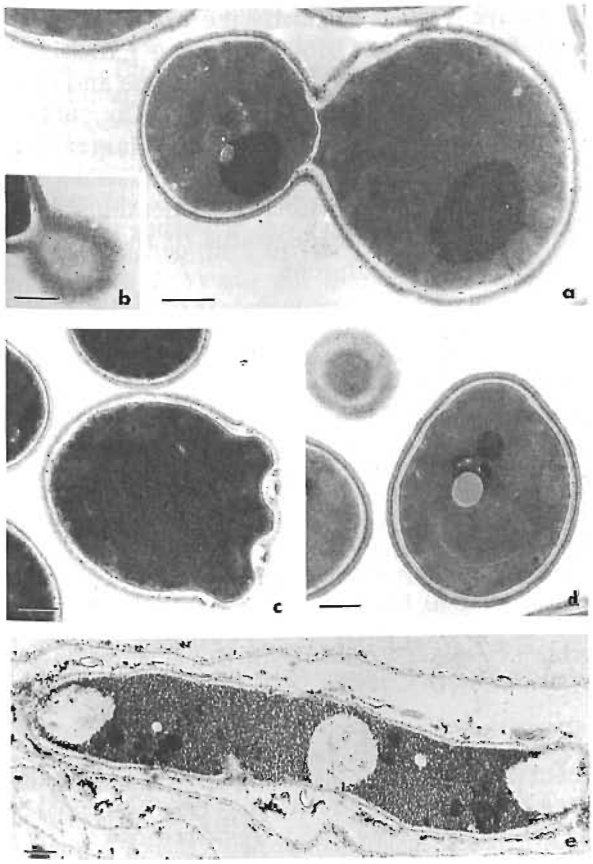

Fig. 4. IgE reactivity of ABPA patient sera 2 (a, b) and 3 (c) to *C. albicans* cell wall clearly visible on tangential section (b). (d) Low background labeling obtained with umbilical cord blood serum on *C. albicans* blastospores. (e) Positive labeling of hyphal cell wall of *A. fumigatus* by incubation with patient serum 2. Glutaraldehyde and osmium tetroxide fixation, araldite embedding, immunogold labeling with mAb specific to human IgE, and 10 nm gold-conjugated goat antimouse IgG. Bar = 0.5 μm.

Shen et al. (28) have identified a 40-kDa major allergen of *C. albicans* among asthmatic patients as an alcohol dehydrogenase. Ishiguro et al. (29) identified various *C. albicans* allergens and purified three components (37, 43, and 46 kDa) from cytoplasmic fractions of *C. albicans* extract. Those three components appeared to be homologous to *S. cerevisiae* aldolase, phosphoglycerate kinase, and enolase, respectively. The protein allergen described by Savolainen et al. (25) had a MW of 46 kDa but represented a major allergen only in a pediatric asthmatic population. They also described a 29-kDa component as an intermediate allergen in children, and it appeared to be a major one in an adult population of atopics (24). In our hands, only two of the seven patients reacted to a component of 29 kDa. Very recently, Akiyama et al. (6) have found a 44-kDa *C. albicans* acid protease to be the allergen responsible for asthma in two atopic patients. In addition to these data, the low IgE reactivity of the ABPA sera in the high MW zone of antigens (relating to *C. albicans* mannan) supports the notion that proteins of lower MW are more prominent determinants of IgE binding. Indeed, allergens from different sources have been characterized over the years and appear to be proteins of small MW. However, in previous reports by Longbottom et al. (30) and Savolainen et al. (25), mannan appeared to elicit an IgE response in a few patients.

The immunoblotting analysis of the IgG subclasses, IgG1–4, reacting to *C. albicans* in the group of ABPA patients, revealed a positive reaction in all the patients except one for IgG1. The IgG4 detection showed reactivity in the high MW zone corresponding to *C. albicans* mannan. This is in accordance with previously published data (29) indicating that a short-term sensitizing antibody, probably IgG4, was elicited by mannans. It appeared that a vast array of *C. albicans* antigens elicits IgG3 production, but not production of the other subclasses, where reactive bands were fewer. IgG3 is the only subclass for which 6/7 ABPA patients reacted to the 43-kDa component. In a recent report, human IgG1, IgG3, and less consistently IgG2, but not IgG4, induced eosinophil degranulation in inflammatory reactions such as bronchial asthma (30). The presence of such specific IgG could represent an additional cause of the symptoms in ABPA patients.

In clinical practice, fungal-sensitive patients often demonstrate immediate skin reactivity to various fungi. This phenomenon may be explained by the presence of cross-reacting components between fungi, as has been shown for *Penicillium notatum*, *A. fumigatus*, and *C. albicans* (31). Cross-reacting components have also been described among atopic children between *Pityrosporum ovale* and *C. albicans* (9). *C. albicans* and *S. cerevisiae* also show cross-reactivity and share common antigens (32–35). In our hands, the adsorption experiments with *C. albicans* extract prevented IgE from binding to *S. cerevisiae* allergens. This result favors the hypothesis of common allergenic components between *C. albicans* and *S. cerevisiae* extracts. This finding was confirmed more recently, and the cell wall mannan was identified as the allergenically cross-reacting component (36). In our study, the IgE-banding pattern against *A. fumigatus* was not altered after adsorption by *C. albicans*, demonstrating the absence of cross-reactivity between these two fungi.

Ultrastructural studies of *C. albicans* have identified as many as seven different layers in the cell wall, depending on the experimental conditions used (37, 38). In the present study, we have been able to localize IgE binding on thin sections of *C.*

albicans by electron microscopy in the mannoprotein-containing layers. Dispersed gold particles can also be detected in the central layer, which contains chitin, β-glucan, and mannoprotein components. Very discrete labeling was observed in the cytoplasm and none in other parts of the blastospores. Since osmium-fixation treatment of cells was used, denaturation of cytoplasmic proteins may have occurred and prevented detection of other IgE-binding components, including the 43-kDa component.

A case report (39) has described allergic bronchopulmonary candidiasis (ABPC), referring to diagnostic criteria similar to ABPA such as immediate cutaneous reactivity, precipitating antibody, and serum IgE specific to *C. albicans*, and isolation of this yeast from the bronchi. The hypothesis of simultaneous occurrence of ABPA and ABPC is mentioned, though not documented. In the present study, eosinophilic pulmonary infiltrates seem to be caused by ABPA. However, one cannot rule out the possibility that pulmonary eosinophilia may have arisen after *C. albicans* hypersensitivity in addition to *A. fumigatus*. The fact that ABPA patients have elevated IgE levels may represent some predisposition for IgE production against other microorganisms similar to *C. albicans*. Therefore, the infiltrates may be related to ABPC. Antibodies belonging to IgE and IgG and reacting against *C. albicans* antigens may participate in the exacerbation of ABPA. Consequently, eradication of *C. albicans* saprophytic growth from the body by antifungals should be considered in the treatment of ABPA patients, as it may improve disease symptoms.

In conclusion, we show here that a group of seven ABPA patients presented IgE antibodies specific to antigens of the yeast *C. albicans* which did not cross-react with *A. fumigatus* allergens. Moreover, it appears that a 43-kDa component, without affinity for ConA and present in yeast as well as mycelium extracts, is a major *C. albicans* allergen in the group of ABPA patients. *C. albicans* may well be implicated in exacerbating disease symptoms in ABPA while participating in pulmonary eosinophilia by some as yet undefined mechanism. Predisposition to colonization by the yeast and persistence of the released antigens after corticosteroid treatment could further induce an IgE immune response. We have also demonstrated by immunoblotting that antibodies belonging to the four subclasses of IgG reacted to various *C. albicans* antigens. In particular, IgG3 was the only subclass to react to the 43-kDa component in 6/7 patients, and this subclass could also participate in the persistent inflammatory reaction recently demonstrated in bronchial asthma (40). Further studies are needed to clarify the exact role played by *C. albicans* in ABPA, including patients presenting at different stages of the disease and receiving different regimens of treatment. Concurrently, a multicentric study, considering a larger group of ABPA cases, should be undertaken in order to determine the possible modifications of the immunologic and clinical status of those patients after antifungal treatment.

Acknowledgments

This work was supported by the Medical Research Council of Canada (MRC) and the Fonds de la Recherche en Santé du Québec (FRSQ). We thank Louis de Repentigny, MD, for critical reading of the manuscript and providing the *A. fumigatus* extract; Guy Delespesse, MD, for expertise in measurement of IgE; Pierre Belhumeur, PhD, for providing the *S. cerevisiae* extract; Simon Garzon, PhD, for electron microscopy studies; and Johanne Blais, BSc, for excellent technical assistance. Eva Roig was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Sainte-Justine Hospital Research Center.

References

1. McCarthy DS, Pepys J. Allergic bronchopulmonary aspergillosis. Clinical immunology. I. Clinical features. Clin Allergy 1971;1:261-86.
2. Malo JL, Pepys J. Allergic bronchopulmonary aspergillosis. In: Weiss EB, Segal MS, Stein M, editors. Bronchial asthma. Mechanisms and therapeutics. 2nd ed. Boston: Little, Brown, 1985:533-42.
3. Longbottom JL, Harvey C, Taylor ML, Austwick PKC, Fitzharris P, Walker CA. Characterization of immunologically important antigens and allergens of *Aspergillus fumigatus*. Int Arch Allergy Appl Immunol 1989;88:185-6.
4. Samuelsen H, Karlsson-Borgå Å, Paulsen BS, Wold JK. Purification of a 20 kDa allergen from *Aspergillus fumigatus*. Allergy 1991;46:115-24.
5. Edge G, Pepys J. Antibodies in different immunoglobulin classes to *Candida albicans* in allergic respiratory disease. Clin Allergy 1980;10:47-58.
6. Akiyama K, Shida T, Yasueda H, Mita H, Yamamoto T, Yamaguchi H. Atopic asthma caused by *Candida albicans* acid protease: case reports. Allergy 1994;49:778-81.
7. Gumowski P, Lech B, Chaves I, Girard JP. Chronic asthma and rhinitis due to *Candida albicans*, epidermophyton, and trichophyton. Ann Allergy 1987;59:48-51.
8. James J, Warin RP. An assessment of the role of *Candida albicans* and food yeast in chronic urticaria. Br J Dermatol 1971;84:227-37.
9. Savolainen J, Broberg A. Crossreacting IgE antibodies to *Pityrosporum ovale* and *Candida albicans* in atopic children. Clin Exp Allergy 1992;22:469-74.
10. Savolainen J, Lammintausta K, Kalimo K, Viander M. *Candida albicans* and atopic dermatitis. Clin Exp Allergy 1993;23:332-9.
11. Pepys J. Skin tests in diagnosis. In: Gell PGH, Coombs RRM, editors. Clinical aspects of immunology. Oxford: Blackwell Scientific, 1975:55.
12. Axelsen NH. Handbook of immunoprecipitation in gel techniques. Scand J Immunol 1983;17 Suppl 10. Oxford: Blackwell Scientific.

13. Sarfati M, Rubio-Trujillo M, Wong K, Rector E, Sehon AH, Delespesse G. *In vitro* synthesis of IgE by human lymphocytes. I. The spontaneous secretion of IgE by B lymphocytes from allergic individuals: a model to investigate the regulation of human IgE synthesis. Immunology 1984; 53:187-96.
14. de Repentigny L, Kilanowski E, Pedneault L, Boushira M. Immunoblot analyses of the serologic response to *Aspergillus fumigatus* antigens in experimental invasive aspergillosis. J Infect Dis 1991;163:1305-11.
15. Savolainen J, Viander M, Einarsson R, Koivikko A. Immunoblotting analysis of concanavalin A-isolated allergens of *Candida albicans*. Allergy 1990;45:40-6.
16. Axelsen NH. Quantitative immunoelectrophoretic methods as tools for a polyvalent approach to standardization in the immunochemistry of *Candida albicans*. Infect Immun 1973; 7:949-60.
17. Lee KF, Buckley HR, Campbell CC. An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. Sabouraudia 1975;13:148-53.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
19. Towbin HT, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350-4.
20. Hawkes R. Identification of concanavalin A-binding proteins after sodium dodecyl sulfate-gel electrophoresis and protein blotting. Anal Biochem 1982;123:143-6.
21. Marquis G, Garzon S, Strykowski H, Auger P. Cell walls of normal and lysozyme-damaged blastoconidia of *Candida albicans*: localization of surface factor 4 antigen and vicinal-glycol staining. Infect Immun 1991;59:1312-18.
22. Reijula KE, Kurup VP, Fink JN. Ultrastructural demonstration of specific IgG and IgE antibodies binding to *Aspergillus fumigatus* from patients with aspergillosis. J Allergy Clin Immunol 1991;87:683-8.
23. Blackley C. Experimental research on the causes and nature of catarrhus aestivus (hay fever or hay asthma). London: Baillière, Tindall and Cox, 1873.
24. Savolainen J, Koivikko A, Kalimo K, Nieminen E, Viander M. IgE, IgA and IgG antibodies and delayed skin response towards *Candida albicans* antigens in atopics with and without saprophytic growth. Clin Exp Allergy 1990; 20:549-54.
25. Savolainen J, Viander M, Koivikko A. IgE-, IgA- and IgG-antibody responses to carbohydrate and protein antigens of *Candida albicans* in asthmatic children. Allergy 1990;45: 54-63.
26. Koivikko A, Kalimo K, Nieminen E, Viander M. Relationship of immediate and delayed hypersensitivity to nasopharyngeal and intestinal growth of *Candida albicans* in allergic subjects. Allergy 1988;43:201-5.
27. Leser C, Kauffman HF, Virchow CSR, Menz G. Specific serum immunopatterns in clinical phases of allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol 1992; 90:589-99.
28. Shen HD, Choo KB, Tang RB, Lee CF, Yeh JY, Han SH. The 40-kilodalton allergen of *Candida albicans* is an alcohol dehydrogenase: molecular cloning and immunological analysis using monoclonal antibodies. Clin Exp Allergy 1991;21:675-81.
29. Ishiguro A, Homma M, Sukai T, Higashide K, Torii S, Tanaka K. Immunoblotting analysis of sera from patients with candidal vaginitis and healthy females. J Med Vet Mycol 1992;30:281-92.
30. Longbottom JL, Brighton WD, Edge G, Pepys J. Antibodies mediating type I skin test reactions to polysaccharide and protein antigens of *Candida albicans*. Clin Allergy 1976;6:41-9.
31. Kumar A, Kurup VP. Murine monoclonal antibodies to glycoprotein antigens of *Aspergillus fumigatus* show cross-reactivity with other fungi. Allergy Proc 1993;14:189-93.
32. Baldo BA, Baker RS. Inhalant allergies to fungi: reactions to bakers' yeast (*Saccharomyces cerevisiae*) and identification of bakers' yeast enolase as an important allergen. Int Arch Allergy Appl Immunol 1988;86:201-8.
33. Greenfield RA, Jones JM. Comparison of cytoplasmic extracts of eight *Candida* species and *Saccharomyces cerevisiae*. Infect Immun 1982;35:1157-61.
34. Hasenclever HF, Mitchell WO. Immunochemical studies on polysaccharides of yeasts. J Immunol 1964;93:763-71.
35. Koivikko A, Kalimo K, Nieminen E, Savolainen J, Viljanen M, Viander M. Allergenic cross-reactivity of yeasts. Allergy 1988;43:192-200.
36. Nermes M, Savolainen J, Kortekangas-Savolainen O. Nitrocellulose-RAST analysis of allergenic cross-reactivity of *Candida albicans* and *Saccharomyces cerevisiae* mannans. Int Arch Allergy Immunol 1995;106:118-23.
37. Garzon S, Marquis G, Montplaisir S, Kurstak E, Benhamou N. Antigenic structure of *Candida albicans*. Electron microscopic localization of polysaccharide and immunodeterminants in the cell wall. In: Kurstak E, editor. Immunology of fungal diseases. New York: Marcel Dekker, 1989:3-36.
38. Marquis G, Montplaisir S, Garzon S, Strykowski H, Auger P. Fungitoxicity of muraminidase. Ultrastructural damage to *Candida albicans*. Lab Invest 1982;46:626-36.
39. Lee TM, Greenberger PA, Soo O, Patterson R, Roberts M, Liotta JL. Allergic bronchopulmonary candidiasis: case report and suggested diagnostic criteria. J Allergy Clin Immunol 1987;80:816-20.
40. Kaneko M, Swanson MC, Gleich GJ, Kita H. Allergen-specific IgG1 and IgG3 through $Fc_{\gamma}RII$ induce eosinophil degranulation. J Clin Invest 1995;95:2813-21.