

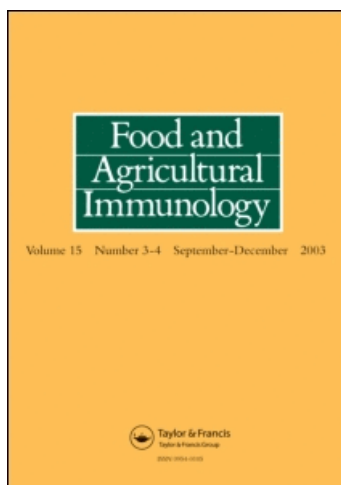
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Modulation of egg white protein allergenicity under physical and chemical treatments

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Hen eggs are one of the most common causes of food allergic reactions. Egg white, which is generally considered more allergenic than egg yolk, has four major allergenic proteins namely, ovomucoid, ovalbumin, ovotransferrin and lysozyme which are defined as immunodominant egg white proteins (EWP). The objectives of the present work were first to evaluate sensitivity to EWP of the population in the region of Fez-Meknes in Morocco, and then to study the effect of acid treatment and heating on the allergenicity of EWP. A cross-sectional study was carried out at the University Hospital in Fez and at analytical laboratories in Meknes in which 446 adults were recruited to establish a serum bank. Sera bank was used to evaluate specific IgE reactivity to EWP and the reactivity of this IgE to native and to heat- and acid-treated EWP. Immunoblots were also carried out to characterise the allergenic components. The results showed that 9.5% of the patients reported allergy to food. Among these patients, 4.2% reported allergy to eggs, 2.5% to peanuts and 0.4% to wheat flour. The study of the effect of temperature and acidity on the allergenicity of EWP showed that more than 50% of sera showed a decrease superior to 30% under heating or acid treatment. With a combination of these treatments, 75% of the sera showed a reduction of more than 30%. By means of immunoblot, we have shown that ovotransferrin and lysozyme are the major allergens for the population studied.

Keywords: allergy; EWP; specific IgE; thermic treatment; acid treatment

Introduction

The prevalence of food allergy in the world is situated between 2 and 4% among adults (Dutau, 2003; Moneret-Vautrin, 2008; Young, Stoneham, Petrukevith, Barton, & Rona, 1994) and from 6 to 8% among children (Dutau, Rancé, Kanny, & Moneret-Vautrin, 1996). Eggs are among the most allergenic foods. Allergy to hen's eggs proves to be the most prevalent; it is the major food allergy among children aged less than 3 years (Eggesbo, Botten, Halvorsen, & Magnus, 2001; Rancé, Kanny, Dutau, & Moneret-Vautrin, 1999). It affects 0.5% of children in the overall population and 5% of atopics (Ford & Taylor, 1982). Allergenic proteins are more present in the egg white than in the yolk (Dutau, 2000). Prevalence of allergy to egg white differs from one country to another. Among children, it is situated between 1 and 14.5% (Julge, Vasar, & Bjorksten, 1997; Kongpanichkul, Vichyanond, & Tuchinda, 1997; Nickel et al., 1997; Ouahidi et al., 2010). Among adults, the rate of

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allergies reaches 12% in Switzerland (Wüthrich, 1993) and Japan (Kosugi et al., 1992).

The main allergens to be found in the egg white are the ovalbumin (54%) Gal d2, ovomucoid (11%) Gal d1 (Langeland, 1982), ovotransferrin (12%) Gal d3 (Langeland & Harbitz, 1983) and lysozyme (3.5%) Gal d4 (Hoffmann, 1983), thus representing 80% of egg white proteins (EWP). Sensitivity of people to different allergens differs according to country and the kind of patients studied (Aabin et al., 1996; Fremont, Kanny, Nicolas, & Moneret-Vautrin, 1997; Urisu et al., 1997).

To this effect, our aim is to evaluate allergy to egg white proteins (EWP) of a large section of patients in the region of Fez-Meknes in Morocco. This study is also concerned with molecular characterisation of the allergenic profile of the patients.

The epitopes can be spread all along the allergenic molecule or basically situated in a specific area. By altering the protein by heating or by another treatment, its immunogenicity will change depending on whether the epitopes are sequential or conformational. The more this equilibrium is in favour to the latter, the more we can expect the decrease of immunogenicity of the protein. This was observed when the egg white was processed by heating, which causes the diminution of its allergenicity (Anet et al., 1985). Other studies did not show the diminution of allergenicity (Besler, Steinhart, & Paschke, 2001; Taylor & Lehrer, 1996). This can be explained by the sensitivity of the patients to some sequential epitopes. The treatments of present-day food manufacturing contain a set of thermal unit and mechanical operations whose goal is to structure, texture and provide a satisfactory conservation of the food item. The modification of food items by means of these processes had led us to study the effect of temperature and of acid processing on the allergenicity of egg white, most often IgE mediated (Eigenmann, 2003), and its components versus the population of the Fez-Meknes region.

Methods

Patients

A transversal study was conducted at the University Hospital Centre of Fez and at the Medical Test Laboratories in Meknes in order to collect serum samples. The people were chosen at random. It should be noted that they had not been sensitised beforehand with regards to the EWP. They were people who came to different medical tests.

A questionnaire was carefully filled out with each patient. The questionnaire contains data relating to age, sex and residence showing possible reactions to food as well as possible treatment regarding hypersensitivity.

Serum collection

After formal consent by the patient, a blood sample was taken and placed in a dry duct which did not contain an anti-coagulant. After centrifugation at 3000 rpm for 5 min, the serum is taken and then stored at -20°C .

Extraction of the egg white proteins (EWP)

A whole chicken egg white was homogenised by stirring for 5 min and then suspended at 1:10 in NaCl 0.9% before centrifugation to eliminate insoluble data. Supernatant was then diluted at 1:5 in NaCl 0.9% and stored at -20°C .

Serologic total IgE determinations

Human sera are incubated overnight at 4°C in a 96-well micro-titration plate. After washing for three times with BBS containing 0.1% of Tween-20 and 0.1% of bovine serum albumin (BSA), the plates are saturated with BSA (0.25%) for an hour at 37°C (200 μl /well). After removal of BSA and washing, the antibody human anti-IgE peroxydase conjugate was added (100 μl /well) in 2 hours at 37°C . The revelation of the immune complex is done after washing by adding chromogene, orthophenylene-diamine (OPD) to the concentration of 0.05%. The reaction is stopped by adding HCl 3M. Absorbance was subsequently determined at 490 nm.

Specific IgE determinations

To immobilise allergens, the EWP were diluted in a tampon PBS at the concentration of 0.5 mg/ml and placed in a volume of 100 μl in each well. The plates were incubated at 4°C overnight. After washing, the plates were conserved at -20°C until use. Human sera, diluted with BBS, are added afterwards; the plate goes through the same stages described earlier for the total IgE.

The determination of specific serical IgE is achieved without prior sensitisation or provocation tests of the patients.

Immunotransfert

An electrophoresis of the EWP is achieved in denatured conditions on polyacrylamide gel. After migration, the gel is either coloured in Coomassie blue or used for immunotransfer. Concerning the latter, the proteins of the gel were transferred on nitro-cellulose membrane. After this transfer, the membranes were cut up and saturated in the presence of BSA at 3% (weight/volume). After washing, each strip was incubated in the presence of a human serum overnight followed up by washing and incubation with anti-IgE peroxydase conjugate. At the end, the reaction is revealed by the incubation of strips in a solution containing 0.05% of diaminobenzidine (DAB) in BBS tampon.

Ethics

This study was approved by the ethics committee of University Hospital Centre of Fez.

Statistical analysis

Statistical analysis was based on the test of student taking $p < 0.05$ as the limit of significant values.

Results

Description of sample

The total number of the patients was 446 adults including 314 women (70.4%) and 132 men (29.6%). Sixteen of the patients at the University Hospital Centre of Fez suffered from urticaria. In Meknes, 217 patients were registered at the test laboratories.

The patients were aged between 17 and 78 years old with an age average of 43.4 ± 1.01 years. The sex ratio is 0.4. Average age for women was 42.5 ± 1.14 and 45.5 ± 2.05 for men.

Reported allergy

The allergy felt is registered thanks to a questionnaire submitted and filled out in the presence of the patients. Of the 446 patients, 283 filled out the questionnaire. The rate of reported allergy, for all foods, is 9.5%. The repartition of the allergy is as follows: 4.2% showed allergy to eggs; 2.5% to peanuts and 0.4% to wheat flour. Egg allergy ranks at the top with the same percentage for women and men (Table 1).

Measurement of total and specific serical IgE

Measurement of total IgE of patients has shown an average of 33.1 ± 1.2 UI/ml. The values vary from 2.8 to 149.2 UI/ml with a rate of 2.25% concerning adults with values higher than 100 UI/ml and 16.25% concerning values between 50 and 100 UI/ml. Dosage of IgE specific to EWP has shown that 34.3% of patients present positive values higher than 2.4 UI/ml: 11.8% have a rate between 20 and 40 UI/ml and 6.2% have values situated between 40 and 100 UI/ml. The average value of these positive values is 14.4 ± 1.1 UI/ml ($n = 139$) varying between 2.4 to 73.5 UI/ml. As regards sex, the average positive value is 13.03 ± 1.09 UI/ml ($n = 97$) concerning women with a maximum of 59.6 UI/ml. As far as men are concerned, the average value is 15.98 ± 2.48 UI/ml ($n = 47$) with a maximum of 73.5 UI/ml.

Effect of temperature and pH on the detection of egg white proteins (EWP) by rabbit IgG

To study the effect of temperature and pH on allergenicity of the EWP, two sets of experimentation were conducted. The first one examined the temperature and pH on the detection of the EWP by a rabbit polyclonal antibody prepared at the laboratory by repeated immunisations by the native egg white. The objective was to identify the

Table 1. Reported allergy to eggs among adults in Fez-Meknes region.

	Adults (283)		
	All	Man ($n = 93$)	Woman ($n = 190$)
Reported allergy	27 (9.5%)	8 (8.6%)	19 (10%)
Reported allergy to egg white proteins	12 (4.2%)	4 (4.3%)	8 (4.2%)

temperature, pH and the amount of time sufficient to inhibit the liaison of the antibodies on the EWP. Figures 1 and 2 represent the achieved findings.

According to Figure 1, the detection of proteins is not modified at 60°C. It is slightly modified during a treatment of 1 hour at 80°C. However, temperature of 100°C highly changes the liaison of antibodies on the heated proteins. We can detect an important increase of the inhibiting effect of the liaison of antibodies on the EWP at 30 min of treatment. In fact, we notice a decrease of 70.7% of liaison of the IgG. This effect slightly decreases simultaneously with the increase of the amount of time of processing.

As regards the acido-basic treatments, we notice that pH acid slightly diminishes the detection of proteins. However, the pH basic tends towards a slight augmentation of detection (Figure 2). In this same figure, we notice that with time, the effect of acid pH is reversed and absorbance tends to augment, indicating a more important detection of proteins by the antibodies.

Effect of temperature processing and acid pH on the allergenicity of human sera

In the second set of experiments, we studied the variation of the allergenicity of the EWP by using human sera whereby the rates of specific IgE rates prove to be important. This allergenicity is studied in terms of optimal parameters of temperature and pH identified in the first set of experimentations.

For this reason, we selected 16 patient cases where specific serical IgE were high (Table 2). These sera were used to determine the liaison of serical IgE on the proteins processed either at temperature (100°C), acidity, or combination of two treatments (Figure 3). The findings show that 68.8% of patients have a high decrease, superior to 30%, in the recognition of proteins when the latter are processed by acidity. We also notice 18.8% of sera (Ala51, L148, 465) have shown a decrease of over 60% in the recognition of proteins. Concerning the proteins processed by temperature, we noticed that 56.2% of patients showed a decrease superior to 30%. There is an overall decrease of 10–70% except for patient no. L125. The latter shows a slight increase of allergenicity to EWP processed at temperature. Regarding the combination of treatments, we notice that 75% of patients showed a decrease of at least 30% of recognition of proteins.

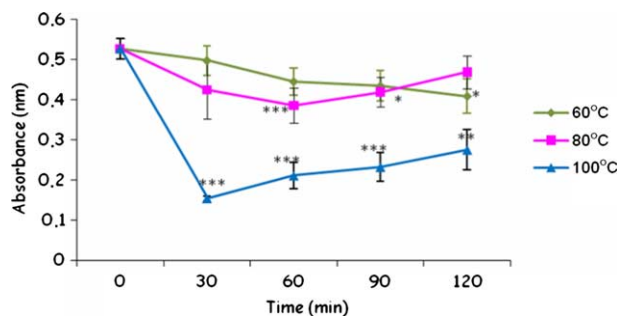


Figure 1. Effect of different temperatures on recognition by rabbit IgG of egg white proteins (EWP) ($N=6$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

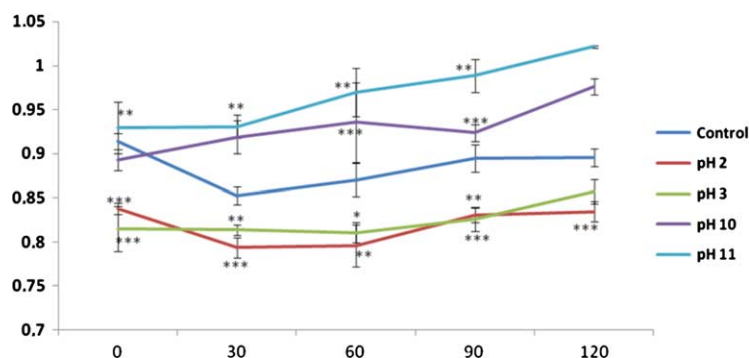


Figure 2. Effect of different pH (pH 2, pH 3, pH 10 and pH 11) on recognition of egg white proteins by rabbit IgG ($N=6$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Immunotransfer

Some sera have been used to achieve an immunotransfer in order to detect allergenic entities which react with specific IgE. In the beginning, we carried out an electrophoresis SDS-PAGE of EWP to acidity and temperature (Figure 4). As regards native proteins, the findings show the presence of five bands corresponding to ovomucin (230 KDa), ovotransferrin (66 KDa), ovalbumin (44.5 KDa), ovomucoid (28 KDa) and lysozyme (14 KDa) (Figure 5). Native EWP or those which were processed by acid or heating were used in immunotransfer to evaluate their detection by the anti-EWP rabbit IgG or by the human serical IgE. The test immunoblot achieved by the anti-EWP rabbit IgG shows the existence of four bands corresponding to ovomucin, ovotransferrin, ovalbumin and lysozyme (Figure 5A). EWP, which underwent an acid treatment shows a reactivity of the rabbit IgG towards the four bands, but with a weaker intensity than test profile. The effect of

Table 2. Serical level of specific IgE anti-EWP.

Patient no.	IgE in UI/ml
Ala 50	53.7
Ala 51	61.9
L148	30.6
L126	39.9
L102	58.8
L150	46
L125	59.6
L136	40.8
L112	73.5
L105	33.7
L82	21.4
L106	35.7
465	46.9
476	21.6
240	23.4
270	23.4

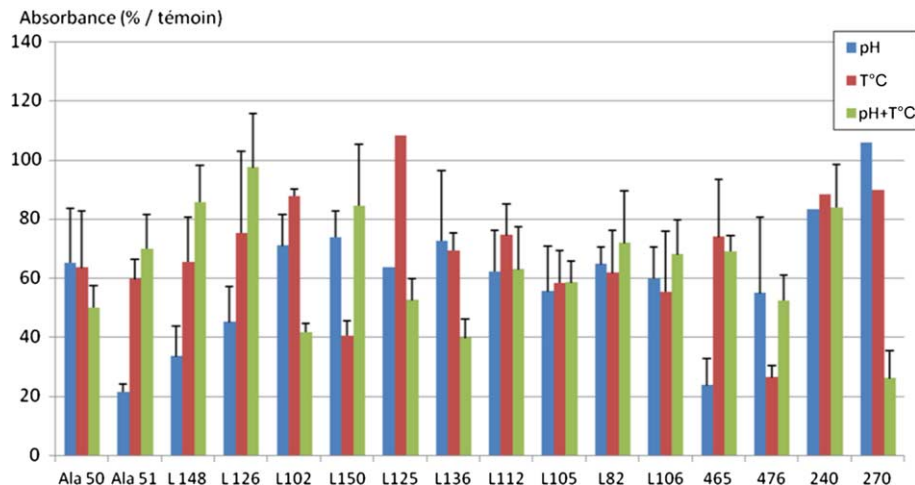


Figure 3. Effect of heat, acidity and a combination of the two treatments on the detection by IgE of egg white proteins. Absorbance represented percentage in relation to control test values. The control test is conducted by using native proteins.

heating EWP shows that the rabbit IgG detect three bands of ovotransferrin, ovalbumin and lysozyme. Temperature partially eliminates the reactivity towards ovomucin (Figure 5C).

The immunoblot test, obtained by the serums of nine patients, shows that the IgE of all the patients detected two bands, indicating a reactivity towards ovotransferrin and the lysozyme. The treatment of the EWP in acid abates the intensity of detection of ovotransferrin and lysozyme by the human IgE (Figure 5B). Heating of EWP eliminates almost completely the reactivity of the IgE towards ovotransferrin and partly the detection of the lysozyme (Figure 5).

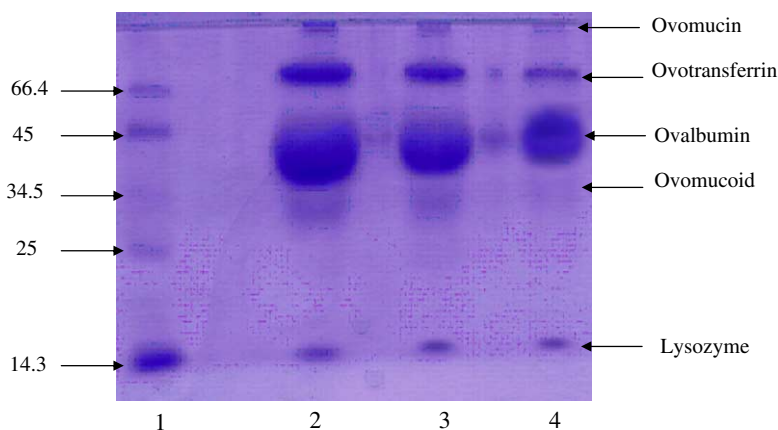


Figure 4. Electrophoresis profile of proteins from native egg white (2), processed by acidity (3), processed by temperature (4). The markers of the molecular weights are dropped in the well (1).

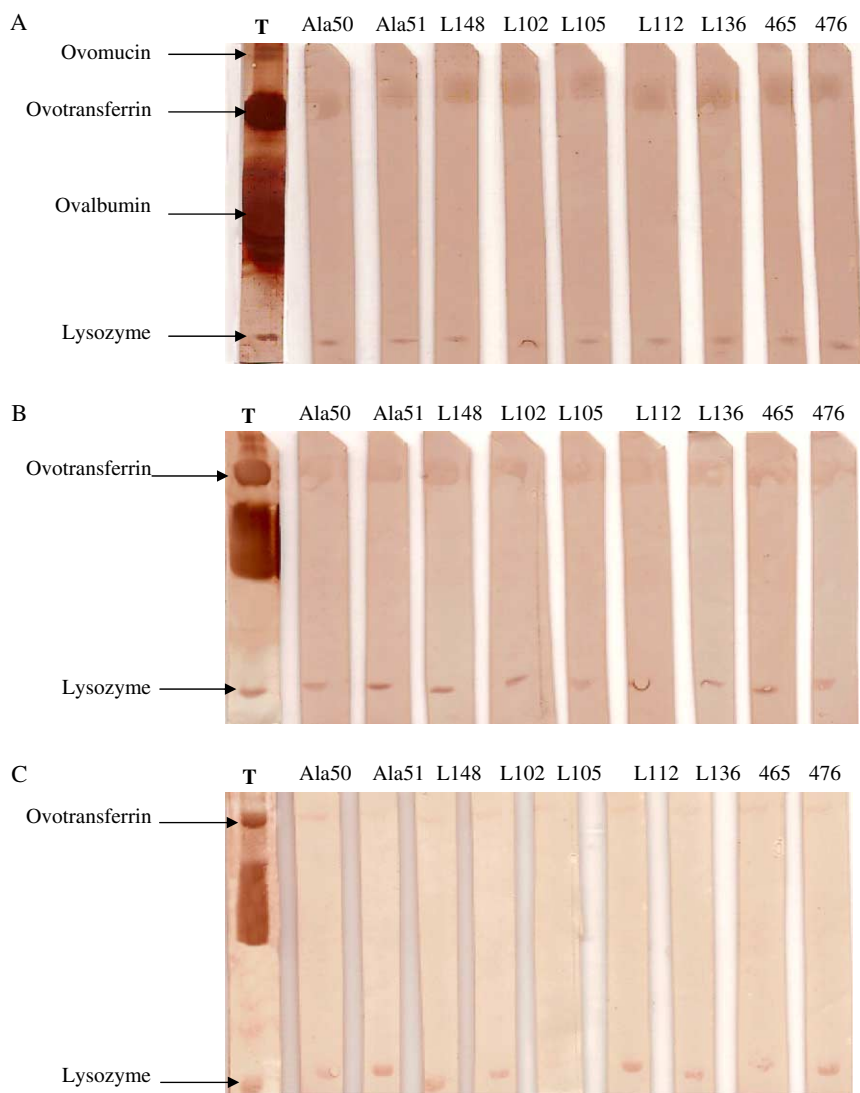


Figure 5. Immunoblot conducted among nine patients (Ala50, Ala51, L148, L102, L105, L112, L136, 465 and et 476). The positive test (T) is prepared by a rabbit anti-EWP serum. (A) Immunoblot with native EWP. (B) Immunoblot with EWP processed by acidity. (C) Immunoblot with EWP processed by temperature.

4. Discussion

The aim of this work was to study the prevalence of allergy to egg white and its characteristics among the population of the Fez-Meknes region in Morocco. The findings have shown that 4.2% of respondent patients have declared that they have sensitivity to eggs. Their sensitivity to eggs is higher than reported earlier (below 2%) (Host & Halken, 1990; Niestijl et al., 1994). However, these reported values were seen to be increasing in France by Moneret-Vautrin who reported 4% for egg

(Moneret-Vautrin, Kanny, & Thevenin, 1998), in Germany where Schäfer reported a value of 3.1% (Schafer et al., 2002), in the USA where the hen eggs had a reported value of 7.2% by means of a questionnaire (Dutau, 2003) and in Singapore a value of 2.7% was reported (Thong, Cheng, Leong, Tang, & Chng, 2007). This variation can be explained by an increase of allergy to eggs among adults. This indicates that the value provided in this study on Fez-Meknes citizens is close to reality and is probably due to the nutrition style where the egg has dominated over the last few years in Moroccan cuisine.

The study of specific IgE values has shown that 11.8% have IgE serical rates that are between 20 and 40 UI/ml. Concerning children, predictive values of allergy were published, allowing the prediction of an allergy by simple determination of the IgE specific to EWP. In fact, Rancé et al. (2003) has shown that a value of 7.5 UI/ml can indicate an allergy to egg. However, among adults, no study has been conducted to identify predictive values. Despite this, it remains that on all the measured values, a considerable percentage of adults have high values of the IgE specific to egg white. This shows that it is very likely that the rate of people who are allergic to egg white will be at least equal, if not superior to, the value 4.2% of allergy reported in this study.

These serums have equally served for the evaluation of their reactivity vis-à-vis proteins constituting the egg white. This immunoreactivity has been studied through two approaches by ELISA and by means of Immunoblot, using two antibodies, the IgG of rabbit and human IgE.

Immunoreactivity of rabbit IgG has been evaluated towards native EWP and EWP having undergone prior heating or acid treatment. The findings have shown that the detection of EWP by the IgG has decreased by 70.7% during heating of EWP at 100°C for 30 min. Such alteration of antibodies liaison by heating EWP is equally observed at the human IgE where 56.2% of examined patients have shown a decrease of at least 30%. This conclusion was reached previously by Anet et al. (1985), Huang, Lemon-Mulé, Bencharitiwong, Sampson, and Nowak-Węgrzyn (2009) and Urisu et al. (1997). Urisu et al. (1997) noticed that 55.3% of the studied allergic patients showed a decrease of the liaison of their IgE on the heated EWP. By means of immunoblot using the rabbit IgG, we noticed that heating of EWP has induced the lowering of the signal of detection of ovomucin, lysozyme and ovotransferrin. The immunoreactivity of the human IgE has revealed that patients react with ovotransferrin and lysozymes. The heating of these leads to a strong decrease of their reactivity with the IgE. In conclusion, the human IgE detects two molecules of EWP, the ovotransferrin and the lysozyme, whose reactivity by antibodies is highly diminished by heating treatment. The presence of two detected major allergens is one of the characteristics of the studied population.

Sensitivity to lysozyme is shared by other countries in the world: Denmark (Aabin et al., 1996), Norway (Holen & Elsayed, 1990), France (Fremont et al., 1997), Japan (Urisu et al., 1997; Yamada et al., 1993) and the USA (Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994; Hoffmann, 1983). Norway, Denmark and the USA share also with our population sensitivity to ovotransferrin.

As regards the diminution of immunoreactivity of antibodies after the heating of the EWP, it was previously detected for ovotransferrin (Mine & Zhang, 2002) and ovomucoid (Julia et al., 2007; Mine & Zhang, 2002). This indicates that the antigenic

epitopes of ovotransferrin and lysozyme detected by the human IgE are predominantly conformational and are modified, at least partially, by heating.

The effect of acid on allergenicity of EWP has shown that detection by the rabbit IgG is very slightly diminished. Conversely, the liaison of the human IgE is highly diminished and we notice that 68.8% of patients show a diminution of at least 30% of their IgE liaison. This indicates that the epitopes detected by the rabbit IgG are not modified by the acid attack. In the opposite, those detected by the IgE are partially conformational. By means of immunoblot, we observed that the ovotransferrin and the lysozyme that are detected by human IgE show a variable sensitivity to the acid attack. The ovotransferrin is more sensitive than the lysozyme. This resistance of lysozyme, noticed previously by Fu, Abbott, and Hatzos (2002), indicates that the diminution of the IgE liaison on EWP is due to a great extent to the loss of the detection of ovotransferrin.

To conclude, the reported allergy to egg among the studied population is 4.2%. This value is confirmed by the specific IgE measurement, indicating a rise of the number of sensitive patients to EWP. Among these antigenic proteins, the ovotransferrin and the lysozyme are the major allergens for the studied patients. By means of immunoblot, we have shown that these molecules are sensitive to heating and to acid processing with an action higher with temperature than the acidity, and a strong resistance of the lysozyme to acid treatment.

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