

Determination of IgG subclass antibodies against wheat flour antigens by an ELISA technique

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We describe the assay conditions for an enzyme-linked immunoassay for the determination of IgG and IgG subclass antibodies in serum to water-soluble wheat flour antigens. The optimal antigen coating concentration was 5 µg/ml for total IgG, IgG₁, IgG₄ and 100 µg/ml for IgG₂. Serial dilutions of test sera were used and commercially available monoclonal mouse anti-human IgG isotype antibodies (as ascites fluid) were diluted 1:500-1:1000. Specific wheat flour antibodies belonging to the IgG₁, IgG₂ and IgG₄ subclasses were detected. Despite the lack of standardized isotype-specific second mouse monoclonal antibodies, the subclass antibody levels between flour-exposed bakers and controls could be compared. We observed significantly higher IgG₁, IgG₂ and IgG₄ subclass antibodies among 23 bakers than among 12 non-exposed controls, but no IgG₃ antibodies were detected. The differences in biological activities of the IgG subclass antibodies may explain the clinical and pathophysiological features for flour-induced occupational allergic diseases among bakers.

Key words: allergen; bakers; ELISA; IgG subclasses; wheat flour.

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Adverse hypersensitivity reactions to flour are well documented (2, 5, 6, 23, 28). Rhinitis and bronchial asthma occur in over 20 % of bakers exposed to flour (16, 29), and flour is the most frequent cause of occupational respiratory disease among bakers in Finland (30). It has been shown that baker's asthma usually is a form of IgE antibody mediated immediate type hypersensitivity reaction to cereal antigens (6, 24). Besides the early Type-I allergic reaction, however, a late response among atopics has been reported to be associated with the presence of heat-stable IgG antibody (21, 22). Raised levels of IgG₄ subclass antibodies have been observed among asthmatics (11, 12), and IgG₄ antibodies have been shown to be capable of binding to basophils and of sensitizing leukocytes to subsequent histamine release (14).

The differences in biological activities of the four subclass antibodies (27) warranted us to

develop a method for determining IgG subclass antibodies to water-soluble wheat flour proteins in the study of occupational respiratory diseases among bakers. This study aimed to investigate the optimal methodological conditions for determining IgG subclass antibodies against water-soluble proteins of wheat flour by an ELISA technique.

MATERIAL AND METHODS

Flour extracts

Wheat flour extracts were made according to the modification of Björkstén et al. (4). 33 g of wheat flour were mixed for 1 h at room temperature (RT) in 100 ml of 0.05 M sodium acetate buffer, pH 3.8. The mixture was centrifuged at 27,000 × g 50 min (Sorvall RC-5B); the super-

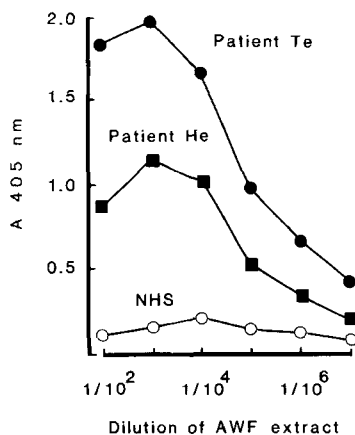


Fig. 1. Effect of antigen coating dilutions of acidic wheat flour extract (stock concentration 10 mg/ml) on the determination of specific total IgG antibodies. Serum dilution 1:100.

natant was collected and designated acidic wheat flour extract (AWF). The extract was concentrated three-fold by ultrafiltration (Amicon, filter YM 5 with cut-off MW 5,000). The protein content of the filtrate was determined according to Lowry et al. (17), adjusted to 20 mg/ml and freeze-dried in 1 ml aliquots.

Antibodies

Mouse monoclonal anti-human IgG subclass antibodies were purchased as ascites fluids from Unipath Ltd. (Bedford, U.K.). Anti-human IgG total (clone 8a4), anti-human IgG₂ (GOM1), anti-human IgG₃ (ZG4) and anti-human IgG₄ (GB7B) were used. All antibodies belonged to the mouse IgG₁ subclass. The specificity of the monoclonal antisera has been tested in a collaborative study and the clones used in the present assay have been found specific and suitable for ELISA (15). Alkaline phosphatase labelled sheep anti-mouse IgG conjugate was obtained from Sigma Chemical Co., USA.

Other reagents

Bovine serum albumin (BSA) and para-nitrophenyl phosphate (pNPP) substrate tablets (5

mg) were purchased from Sigma Chemical Co., USA. Phosphate buffered saline (PBS) pH 7.4 was used as coating buffer, PBS-0.05% Tween 20-1 mM MgCl₂·6H₂O-0.1% BSA as diluent for test sera and antisera, 10% diethanolamine, pH 9.8, was used as substrate buffer and 3 M NaOH as stop solution. Washing buffer was PBS 0.05% Tween 20.

Patient sera

For determining optimal assay conditions, sera from two patients clinically suffering from flour-related allergic symptoms were used.

Patient "Baker He" had a 7-year exposure history to flour, mainly wheat and rye. He had a 6-year history of rhinitis and sneezing at the work place. Standard skin tests to flours were negative and no specific IgE antibodies to flour could be observed by the RAST technique. After exposure to wheat flour dust, mucous swelling, secretion of the nose and sneezing was observed.

The second patient "Baker Te" had a 15-year history of exposure to wheat and other flour dusts. She had no history of atopy. During the last year she had experienced work-related shortness of breath and wheezes but no rhinitis or conjunctivitis. The concentration of serum IgE was normal and no specific IgE antibodies to different flours could be detected by the RAST technique. There was no bronchial hyper-reactivity to histamine. Due to strong demographism, skin testing results to flour extracts were inconclusive. During exposure to wheat flour dust no decrease in peak expiratory flow values were observed.

The study subjects were 23 flour-exposed bakers (10 men, 13 women, age range 17 to 61, median 33 years). They had exposure histories of 1.5 to 31 years (median 12 years) and were admitted to our institute for the evaluation of allergic complaints. Twelve unexposed persons (6 men, 6 women) were used as controls.

IgE-measurements

The total IgE and the flour-specific IgE was measured using Phadezym Prist and Phadezym

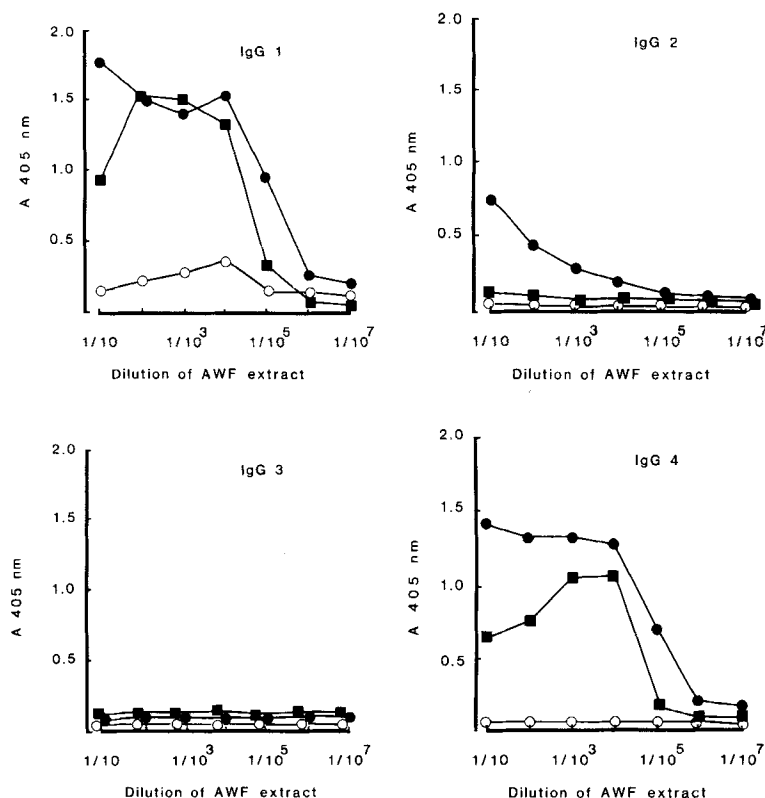


Fig. 2. Effect of antigen coating dilutions of acidic wheat flour extract (stock concentration 10 mg/ml) on the determination of specific IgG subclass antibodies. The sera of bakers Te (●) and He (■) and NHS (○) were diluted 1:100 for total IgG and 1:30 for IgG₁, IgG₂, IgG₃ and IgG₄.

RAST assay, respectively (Pharmacia, Uppsala, Sweden).

IgG subclass enzyme-immunoassay

The lyophilised flour extract was dissolved into PBS, pH 7.4 to a stock solution of 10 mg/ml. The extract was filtered through Millex 0.22 µm filter (Millipore Co.) before dilution. Flat-bottomed polystyrene microtiter plates (Microstrips, Eflab Ltd., Finland) were coated with 125 µl of antigen extract at room temperature over night. Sera were added to the wells in the following order: 1) 100 µl of serum were incubated over night at dilutions as indicated, and 2) 100 µl of mouse monoclonal anti-human total IgG and IgG subclass antibodies (dilution

1:1000, except for anti-human IgG₂ which was diluted 1:500) were incubated for 2 h at room temperature. This was followed by addition of 100 µl alkaline phosphatase conjugated sheep anti-mouse IgG (dilution 1:1000) and further incubation of 1 h. Alkaline phosphatase activity was determined using substrate concentration of 1 mg/ml and 30 min incubation time. The reaction was stopped with 3M NaOH. Between each step the plates were washed three times for 5 min each time with 200 µl washing buffer. All incubations were carried out on a shaker. The absorbances were read at 405 nm using a Titer-tek-Multiscan photometer (Flow Laboratories, England). The absorbances measured were changed to arbitrary units using the reference serum according to the formula

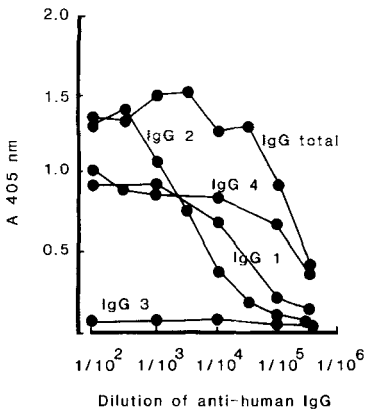


Fig. 3. Effect of dilutions of monoclonal anti-human IgG subclass antibodies. Microplates were coated with acidic wheat flour extract, 5 µg/ml for total IgG, IgG₁ and IgG₄ assays and with 100 µg/ml for IgG₂ and IgG₃ assays. The serum (baker Te) was diluted 1:100 for total IgG and 1:30 for IgG₁, IgG₂, IgG₃ and IgG₄.

$$\frac{A(\text{sample}) - A(\text{blank}) \times 100}{A(\text{reference}) - A(\text{blank})}$$

The final titers were read from a graph where these units were plotted against the corresponding serum dilutions. The titer of antibody corresponds to the value of 10 arbitrary units (see Fig. 4, IgG₄).

In each assay a selected positive control serum was included. The between-days coefficients of variations were: for total IgG 12 % (number of runs: 7), IgG₁ 15 % (*n* = 5), IgG₂ 26 % (*n* = 9) and IgG₄ 16 % (*n* = 10).

Inhibition studies

Two positive sera were tested in the presence of antigen competition with five different antigen

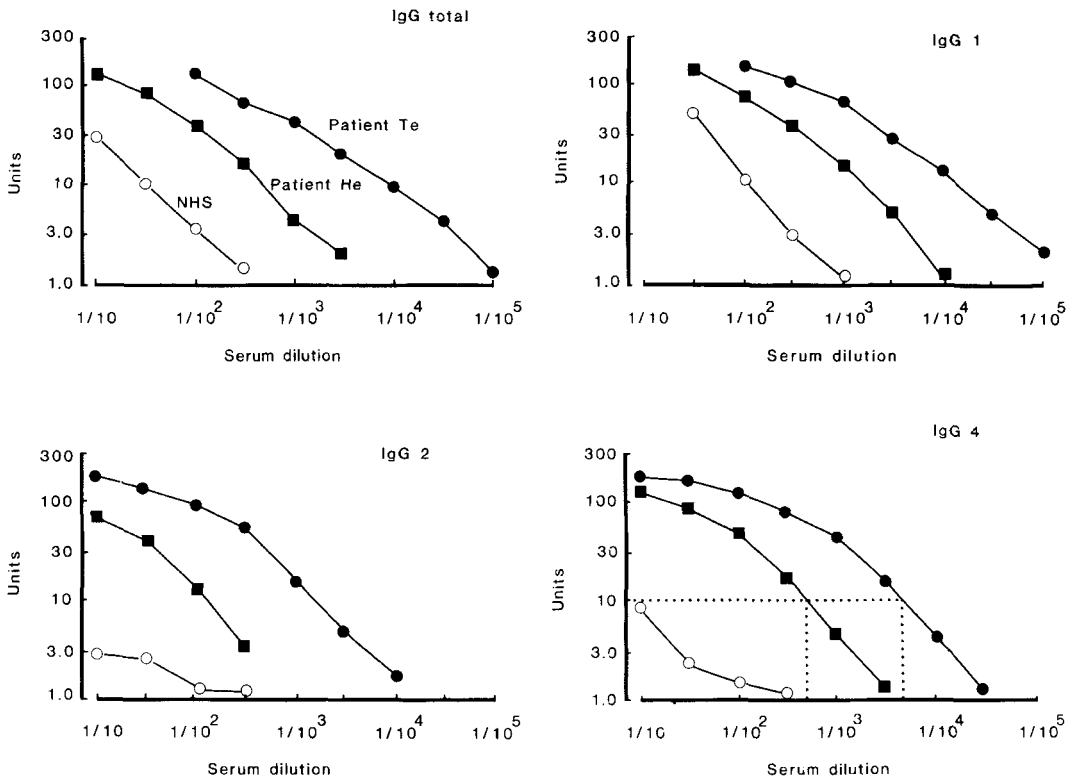


Fig. 4. Effect of serum dilution on the determination of total IgG, IgG₁, IgG₂ and IgG₄ subclass antibodies of bakers Te (●) and He (■) and NHS (○) against acidic wheat flour extract (coating as in Fig. 3). Mouse anti-human IgG tot, IgG₁ and IgG₄ were diluted 1:1000 and anti-human IgG₂ was diluted 1:500.

concentrations (0.01–100 $\mu\text{g/ml}$). The sera (dilution chosen from the top part of the serum dilution curve) were incubated for 60 min at 37°C with the antigen, followed by immunoassay as described. The percentage of inhibition was compared with the results obtained in the absence of competing antigens.

Statistics

The antibody titers among bakers and controls were compared by using the Mann-Whitney-Wilcoxon rank sum test.

RESULTS

Polystyrene microtiter plates gave a low background binding (blank values from 0.030 to 0.060) and good uniformity of measurements in all wells, CV = 8.5% within the plate ($n = 95$).

The optimal concentration of AWF bound to the wells was studied by dilution of the coating antigen. For total IgG antibody determinations (Fig. 1), the optimal concentration of coating antigen was 5 $\mu\text{g/ml}$ for both reference patients. With this antigen coating, normal human serum (NHS) gave a low absorption. The optimal

coating concentration was chosen from the upper part of a titration curve, near the saturation point. For the determination of IgG subclass antibodies against AWF the optimal coating concentrations were 5 $\mu\text{g/ml}$ for IgG₁ and IgG₄, and 100 $\mu\text{g/ml}$ for IgG₂ (Fig. 2). In neither of the patients could we detect any IgG₃ antibodies.

Fig. 3 shows the effect of the dilution of monoclonal anti-human IgG subclass antibodies on the absorption of 405 nm. Based on these results, the dilutions used for subsequent assays were 1:1000 for determination of total IgG, IgG₁, IgG₄ and 1:500 for IgG₂.

Serial dilution of serum was used to determine the levels of total IgG and subclass antibodies to AWF. The absorbances measured were first changed into arbitrary units using a reference serum (see Material and Methods). Fig. 4 shows the effect of serum dilution compared with the calculated arbitrary units of antibody. The final antibody titers were read from the linear part of the serum dilution curve, and expressed as the serum dilution corresponding to 10 units (shown for IgG₄ by the dotted lines in Fig. 4).

The sensitivity of the assay was examined by diluting serum from two patients. For the most positive serum (patient Te) antibodies were detected at serum dilutions up to 1:1000000 in total IgG assays, in dilution from 1:100 to 1:100000 in IgG₁ assays, in dilutions from 1:10 to 1:10000 in IgG₂ assays, in dilutions ranging from 1:30 to 1:100000 in IgG₄ assays (Fig. 4).

We tested the specificity of the IgG₄ antibody binding by antigen competition. Fig. 5 shows the inhibition of IgG₄ binding to the solid phase for two bakers' sera. The IgG₄ binding of baker Te serum was inhibited to 50% with 0.06 $\mu\text{g/ml}$ of competing antigen extract, whereas the serum of baker He was inhibited to 50% with an antigen concentration of 0.02 $\mu\text{g/ml}$. We also similarly tested the inhibition of total IgG, IgG₁ and IgG₂ subclass binding (data not shown).

We determined the levels of total IgG, IgG₁, IgG₂, IgG₃ and IgG₄ antibodies against AWF in 23 bakers and 12 non-exposed controls (Fig. 6). Significantly higher levels of specific total IgG,

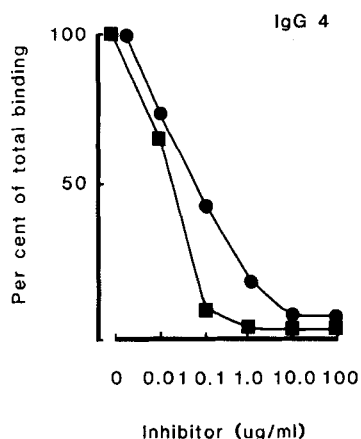


Fig. 5. Specific inhibition by wheat flour extract in the IgG₄ antibody assay. Microplates were coated with 5 $\mu\text{g/ml}$ acidic wheat flour extract. Two bakers' sera Te (●), He (■) were diluted 1:100 with PBS containing 0.01–100 $\mu\text{g/ml}$ wheat flour extract and incubated for 1 h at 37°C, and then introduced into wells.

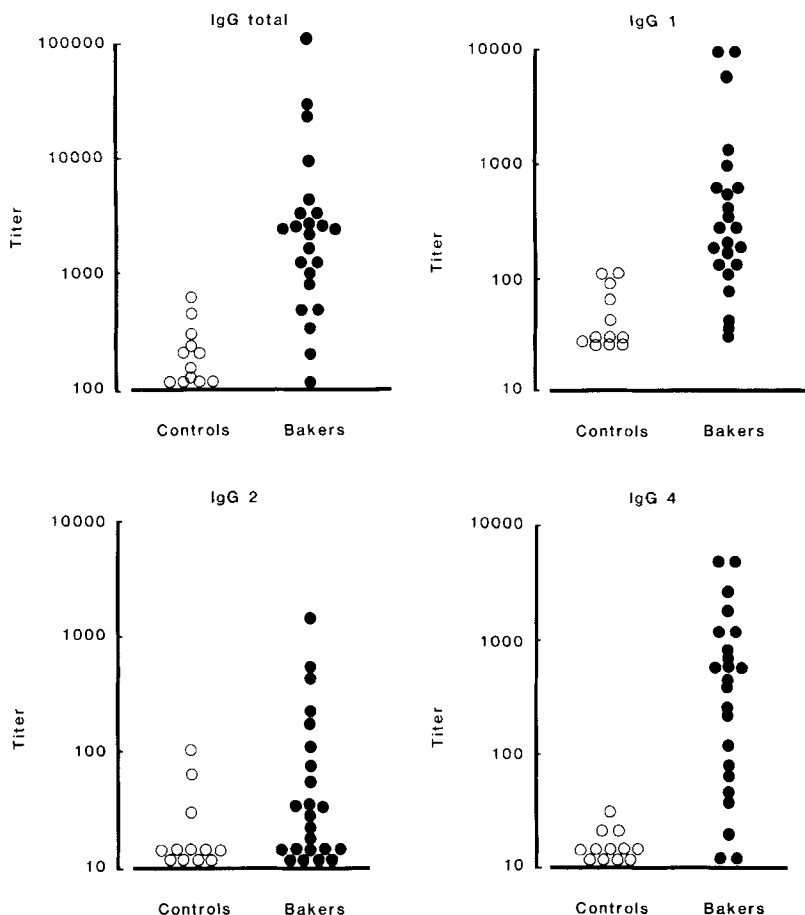


Fig. 6. Distribution of specific total IgG and IgG₁, IgG₂ and IgG₄ subclass antibodies against wheat flour proteins among 12 controls and 23 bakers.

IgG₁ and IgG₄ antibodies ($P < 0.01$) were detected among bakers than among controls. No antibodies belonging to the IgG₃ subclass could be detected.

DISCUSSION

The purpose of this study was to develop an assay for the determination of IgG subclass antibodies against soluble wheat flour proteins. The use of purified antigens in immunoassays is recognized as being beneficial when specific antibodies are measured. However, wheat flour is a complex mixture and several of its components have been found to react with IgE posi-

tive sera (5). The purification of antigens in this assay was limited to remove small molecules (mw below 5000) by ultrafiltration. Studies on wheat flour antigen composition using polyacrylamide gel electrophoresis and immunoblotting techniques and IgG subclass antibody specificities are currently in progress.

The optimal antigen-coating concentration varied from 5 µg/ml (total IgG, IgG₁ and IgG₄) to 100 µg/ml (IgG₂) when concentrations were chosen near the point of antigen saturation at which absorbed proteins are stably bound throughout the assay (7). It seems that coating with 100 µg/ml solution in IgG₂ assay increases the absorption of components against which

IgG₂ antibodies are reacting. The dilution 1:500 to 1:1000 of the monoclonal IgG subclass antibodies proved to be adequate. All the monoclonal antibodies were of the same IgG subclass (mouse IgG₁) giving a similar binding of the enzyme-labelled rabbit anti-mouse antibodies.

Antibodies belonging to the IgG₁, IgG₂ and IgG₄ subclasses could be detected both among bakers and non-exposed controls. Due to the lack of standardized isotype-specific second antibodies, the amount of different isotypes can only be estimated and the proportions of different subclass antibodies among bakers cannot be absolutely compared. However, a comparison between antibody levels within each subclass can be made between bakers and non-exposed controls.

In general, specific antibody responses to protein antigens predominantly belong to the IgG₁ subclass. Protein antigens also stimulate the synthesis of IgG₂, IgG₃ and IgG₄ antibodies but often to a lesser extent than IgG₁. When determining the antibodies against wheat flour extract, the most commonly occurring reactivity was found in IgG₁ antibodies. The serum which showed IgG₂ and IgG₄ activity was usually found positive also in the IgG₁ assay.

It has been observed that IgG₂ subclass responses develop to carbohydrate antigens, and that they develop slowly, and also, that they are detected only at the late stages of certain diseases (20). The presence of IgG₂ subclass antibodies in only a minority of the bakers compared with controls may be explained by the antigen composition of wheat flour.

Although IgG₃ subclass antibodies are produced to a number of exogenous viral and parasitic antigens (3, 9, 26), IgG₃ antibodies to wheat flour were not detected among our bakers. This could be explained by the observations that IgG₃ antibodies are produced in an early antibody response to proteins, and that IgG₃ has a shorter half-life than the other isotypes (19). The present results suggest that specific IgG₃ isotypes are underrepresented among bakers.

IgG₄ represents only 4% of total IgG but it may account for a remarkable portion of specific antibodies. IgG₄ subclass antibodies were de-

tected in the majority of bakers. This is in keeping with observations that specific IgG₄ subclass responses occur after exposure to a number of foods, pollens, venoms and other protein antigens (1, 8, 18). This response has also been influenced by antigen dosage, route, and chronicity of exposure, as well as the age and atopic status of the individual (25). Aalberse et al. (1) have suggested IgG₄ to be the dominant IgG subclass following chronic antigen stimulation. Among our 23 bakers, nine had specific IgE antibodies to one or several flours as determined by the RAST technique. This suggests that the mechanisms eliciting IgG₄ and IgE responses may be interrelated. A close association between the levels of IgG₄ and IgE antibodies in allergic patients has been observed (13) and both types of antibodies, when bound to basophils, may stimulate histamine release upon antigen contact (10). We have correlated the IgG subclass antibody levels among bakers to various clinical variables including IgE levels, duration of asthmatic or rhinitis symptoms, skin testing results, peripheral blood eosinophil levels, bronchial histamine hyperreactivity and responses to wheat flour challenge tests (Tiikkainen & Klockars, to be published). Also, the possible clinical and pathophysiological significance of IgG subclass antibodies compared with specific IgE antibodies to flour for the development of occupational rhinitis and asthma is currently being investigated.

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REFERENCES

1. Aalberse, R. C., van der Gaag, R. & van Leeuwen, J.: Serological aspects of IgG₄ antibodies. I. Prolonged immunization results in an IgG₄-restricted response. *J. Immunol.* 103, 722-726, 1983.
2. Baldo, B. A. & Krilis, S.: Hypersensitivity to inhaled flour allergens. Comparison between cereals. *Allergy* 35, 45-56, 1980.
3. Beck, C. F. & Heiner, D. C. Selective immunoglobulin G₄ deficiency and recurrent infections of the respiratory tract. *Am. Rev. Respir. Dis.* 124, 94-96, 1981.
4. Björkstén, F., Backman, A., Järvinen, K. A. J. et al.: Immunoglobulin E specific to wheat and rye flour proteins. *Clin. Allergy* 7, 473-483, 1977.

5. Blands, J., Diamant, B., Kallos, P., Kallos-Deffner, L. & Löwenstein, H.: Flour allergy in bakers. *Int. Arch. Allergy Appl. Immunol.* **52**, 392-406, 1976.
6. Block, G., Tse, K. S., Kijek, K., Chan, H. & Chan-Yeung, M.: Baker's asthma. Studies of the cross-antigenicity between different cereal grains. *Clin. Allergy* **14**, 177-185, 1984.
7. Cantarero, L. A., Butler, J. E. & Osborne, J. W.: The adsorptive characteristics of proteins for polystyrene and their significance in solid-phase immunoassays. *An. Biochem.* **105**, 375-382, 1980.
8. Devey, M. E., Wilson, D. V. & Wheeler, A. W.: The IgG subclasses of antibodies to grass pollen allergens produced in hay fever patients during hyposensitization. *Clin. Allergy* **6**, 227-236, 1976.
9. El Amin, E. M., Wright, E. P. & Vlug, A.: Characterization of the humoral immune response in Sudanese leishmaniasis: specific antibody detected by class- and subclass-specific reagents. *Clin. Exp. Immunol.* **64**, 14-19, 1986.
10. Fagan, D. L., Slaughter, C. A., Capra, J. D. & Sullivan, T. J.: Monoclonal antibodies to immunoglobulin G₄ induce histamine release from human basophils in vitro. *J. Allergy Clin. Immunol.* **70**, 399-404, 1982.
11. Gwynn, C. M., Morris-Smith, J., Leon Leon, G. & Stanworth, D. R.: Role of IgG₄ subclass in childhood allergy. *Lancet* **i**, 910-911, 1978.
12. Gwynn, C. M., Morrison-Smith, J., Leon Leon, G. & Stanworth, D. R.: IgE and IgG₄ subclass in atopic families. *Clin. Allergy* **9**, 119-123, 1979.
13. Hammarström, L. & Smith, C. I. E.: Immunoglobulin subclass distribution of specific antibodies in allergic patients. *Allergy* **42**, 529-534, 1987.
14. Hindocha, P. & Wood, C. B. S.: Histamine release from human leucocytes by IgG₄ subclass in the sera of allergic children. *Allergy* **40**, 523-528, 1985.
15. Jefferis, R., Reimer, C. B., Skvaril, F., et al.: Evaluation of McAb having specificity for human IgG subclasses. *Immunol. Letters* **10**, 223-252, 1985.
16. Järvinen, K. A. J. & Piriälä, V.: Unsuitability of bakery work for a person with atopy: a study of 234 bakery workers. *Ann. Allergy* **42**, 192-194, 1979.
17. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-285, 1951.
18. Merrett, J., Burr, M. L. & Merrett, T. G.: A community survey of IgG₄ antibody levels. *Clin. Allergy* **13**, 397-407, 1983.
19. Morell, A., Terry, W. D. & Waldman, T. A.: Metabolic properties of IgG subclasses in man. *J. Clin. Invest.* **49**, 673-680, 1970.
20. Olsson, I., Hammarström, L., Smith, C. I. E., Hovmark, A. & Åsbrink, E.: IgG subclass distribution of specific antispirochetal antibodies in *Ixodes ricinus* borne-Borrelia spirochetosis. *Clin. Exp. Immunol.* **69**, 618-623, 1987.
21. Parish, W. E.: Short-term anaphylactic IgG antibodies in human sera. *Lancet* **ii**, 591-592, 1970.
22. Pepys, J., Parish, W. E., Stenius-Aarniala, B. & Wide, L.: Clinical correlations between long-term (IgG S-TS) anaphylactic antibodies in atopic and non-atopic subjects with respiratory allergic disease. *Clin. Allergy* **9**, 645-658, 1979.
23. Prichard, M. G., Ryan, G. & Musk, A. W.: Wheat flour sensitization and airways disease in urban bakers. *Br. J. Ind. Med.* **41**, 450-454, 1984.
24. Prichard, M. G., Ryan, G., Walsh, B. J. & Musk, W.: Skin test and RAST responses to wheat and common allergens and respiratory disease in bakers. *Clin. Allergy* **15**, 203-210, 1985.
25. Rowntree, S., Platts-Mills, T. A. E., Cogswell, J. J. & Mitchell, E. B.: A subclass IgG₄-specific antigen-binding radioimmunoassay (RIA): Comparison between IgG and IgG₄ antibodies to food and inhaled antigens in adult atopic dermatitis after desensitization treatment and during development of antibody responses in children. *J. Allergy Clin. Immunol.* **80**, 622-630, 1987.
26. Scott, M. & Goss-Sampson, M.: Restricted IgG isotype profiles in *T. cruzi* infected mice and Chagas disease patients. *Clin. Exp. Immunol.* **58**, 372-379, 1984.
27. Spiegelberg, H. L.: Biological activities of immunoglobulins of different classes and subclasses. *Adv. Immunol.* **19**, 259-293, 1974.
28. Sutton, R., Skerriitt, J. H. & Baldo, B. A.: The diversity of allergens involved in bakers asthma. *Clin. Allergy* **14**, 93-107, 1984.
29. Thiel, H. & Ulmer, W. T.: Bakers' asthma: Development and possibility for treatment. *Chest* **78**, 400-405, Supplement, 1980.
30. Vaaranen, V., Vasama, M. & Alho, J.: Occupational diseases in Finland. New cases of occupational diseases reported to the Finnish Register of Occupational Diseases. Reviews 11. Institute of Occupational Health, Helsinki 1985.

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