Acta Derm Venereol (Stockh) 1992; Suppl. 176: 45–48

Study of Immune-responsiveness to Wheat Antigen by IgG, IgA, and IgE Immunoblotting with Sera from Patients with Atopic Dermatitis

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To investigate the immune mechanism underlying the IgE-mediated hypersensitivity to food antigens, wheat-flour proteins were extracted in mild condition, and IgG antibodies were detected by the ELISA method. Atopic dermatitis patients who had high scores for IgE-RAST were shown to have increased levels of IgG antibodies to wheat proteins. To define the allergenic polypeptides or epitopes in wheat proteins, each patient's serum was subjected to determination of IgG, IgA, and IgE antibodies to each protein component, using a highly sensitive immunoblotting method. Low molecular weight polypeptides bind specifically IgG, IgA, and IgE antibodies in serum from atopic dermatitis patients. Thus, there are specific components or epitopes in wheat proteins which are closely related to the disease states. ***Key Words: Atopic dermatitis, Food allergy; Wheat antigen; IgE-RAST; Immunoblotting.***

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INTRODUCTION

The etiology of atopic dermatitis (AD) remains unknown. Several immunologic abnormalities have been noted but the underlying defect has not yet been established. Recent studies have suggested a central role for an IgE-mediated mechanism, and a correlation of disease flares on exposure to various food antigens. However, the immune mechanisms underlying such hypersensitivity to food antigens, espeially wheat, has not yet been investigated. At our clinics, there are many patients with AD that might be caused by wheat-flour hypersensitivity. In the present study, allergenic wheat proteins were extracted from wheat flour, and IgG antibodies to the extracted wheat proteins were measured by the ELISA method in sera of wheat-RAST-positive AD patients as well as in sera of healthy controls. Moreover, IgG, IgA, and IgE antibodies directed to each protein component were examined by the immunoblotting method.

Our findings indicated that antibody response which might be the important background in the expression of AD varies among patients and healthy controls, and that there are specific components or epitopes in wheat proteins that are closely related to the disease states.

MATERIALS AND METHODS

Sera

Test sera were obtained from 15 patients allergic to wheat. The IgE-RAST scores were more than 3 points, and all had been suffering from severe AD. They were diagnosed according to Dr Rajka's criteria, including itching, chronic course, atopic history, and typical atopic lesions. Control persons were negative for wheat- specific IgE antibodies as well as for total IgE antibodies.

Extraction of wheat flour proteins

Wheat flour was defatted by extraction with diethyl ether, washed, and then air-dried. The proteins were extracted from defatted powder (1.0 g) with UTC solution (7 M Urea, 20 mM 2-mercaptoethanol in 0.025 Tris-citrate buffer pH 8.6) by stirring for 1 h at room temperature. The extracts were centrifuged, and the supernatant was dialysed against 20 mM phosphate buffer, pH 7.2, in dialysis tubing with a pore limit of 3500 MW. Samples were lyophilized and resuspended in 10 ml of UTC solution. After filtration through 0.22 θm nitrocellulose filters, samples were stored at -20°C until use.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The extracted sample was dissolved in 1 ml of 50 mM Tris-HCl containing 2% SDS, 5% 2-mercaptoethanol, 20% glycerol, and 0.05% bromphenol blue, and then boiled for 2 min. 10–20% gradient gels were used for SDS-PAGE. After the completion of electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes, and assayed for detection of antibodies in patients' sera. For detection of specific IgG and IgA antibodies to wheat proteins, patients' sera were used at 1:20 dilution in blocking solution (PBS-Tween 20, 0.5% skim milk). The secondary antibody, biotinylated goat anti-human IgG or IgA (1:500 dilution), respectively, was incubated at 37°C for 60 min. Avidin-bound horseradish peroxidase (1:1000) was then added, and the immune-complexes were visualied by applying 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB)/0.045% H_2O_2.

For detection of specific IgE, sera were not diluted, and the secondary antibody was biotinylated goat anti-human IgE (1:500).

Enzyme-linked immunoassay

The extracted wheat proteins were fixed to the bottom of 96-well microtiter plates. 100 θl of polypeptides (0.1 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated overnight at 4°C. Non-specific binding sites were blocked with 2% bovine serum albumin (BSA) in PBS, by incubating for at least 1 h at 37°C. Serum was diluted (1:100) in PBS containing 0.2% BSA and 0.05% Tween-20. The antibody dilution was added to the microtitre wells and allowed to incubate for 1 h at 37°C. After washing with PBS, peroxidase-conjugated goat anti-human IgG (1:4000) was added to the wells for 1 h at 37°C. After washing with PBS, *o*-phenylenediamine was added to the wells. After 10 min at 37°C, the absorbance in each well was determined at 490 nm using a microtitre reader.

RESULTS

The SDS-gradient PAGE pattern of the wheat preparation is shown in Fig. 1. Lane A is molecular weight marker pol-

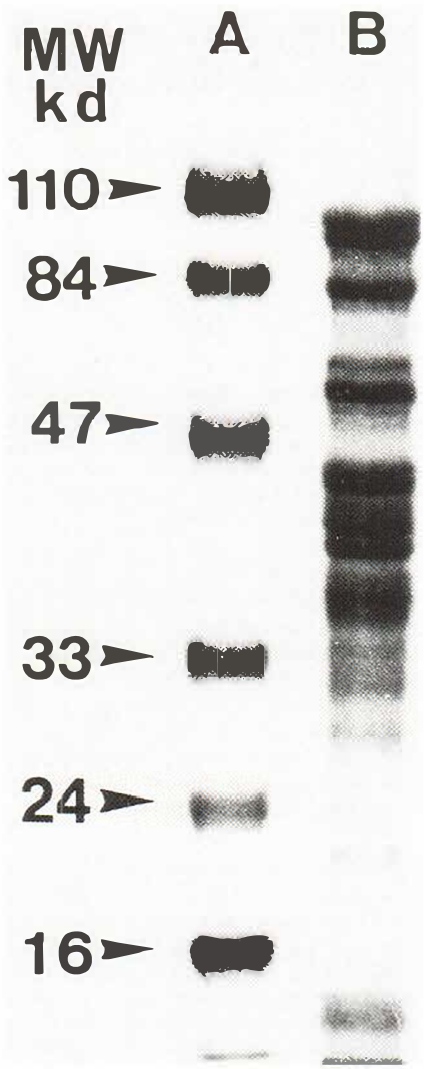

Fig. 1. SDS-PAGE pattern of extracted wheat flour proteins. *A:* molecular weight marker. *B:* wheat flour proteins.

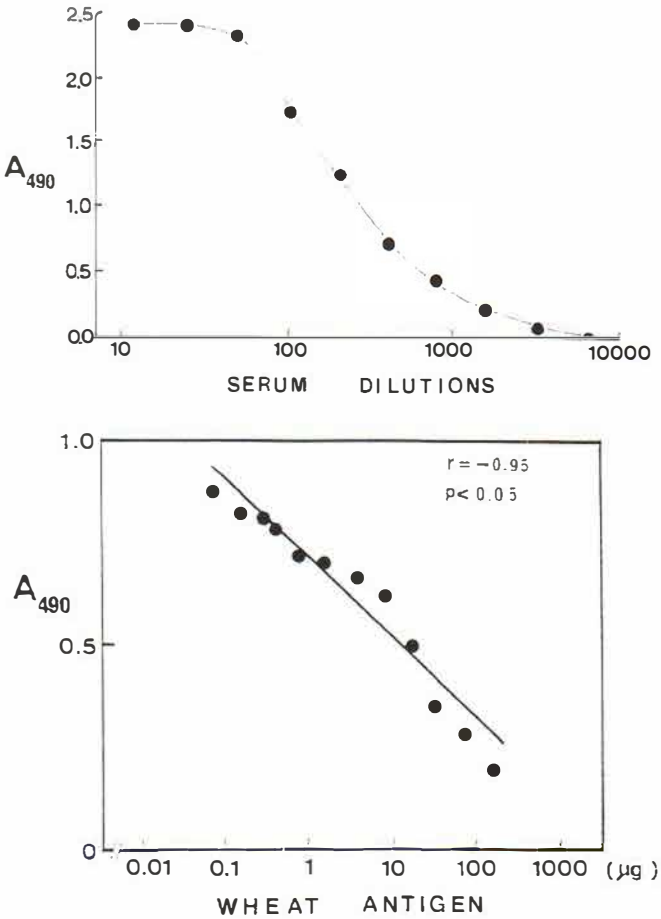

Fig. 2. (*a*) Enzyme-linked immunoassay for IgG antibodies to wheat proteins – a standard curve. (*b*) Inhibition test for ELISA sytem. Addition of wheat proteins to our established ELISA sytem inhibited IgG binding to the fixed protein dependent on the protein concentration.

ypeptides. Lane B is wheat polypeptide pattern. Wheat proteins could be resolved by SDS-PAGE into many polypeptides of molecular weights between 12 and 100 kDa. The staining pattern was then analysed by densitometer. Polypeptides between 30 and 40 kDa of molecular weight were found in much greater quantity than the others.

In the next series of experiments, serum IgG levels specific for wheat antigens were determined by ELISA methods. As shown in Fig. 2*a*, a standard curve was drawn using pooled sera diluted 1:10 to 1:10000. The ELISA unit was set at 1000 units/ml as reference in the pooled normal serum. Then, specificity of reaction to wheat proteins was determined by inhibition assay (Fig. 2*b*). Addition of increasing amounts of wheat protein resulted in a specific reduction of the reaction. Thus, this system is highly specific to wheat proteins.

Using this ELISA system, IgG antibodies were determined in sera of AD patients as well as in healthy controls (Fig. 3). Elevated levels of IgG antibodies were detected in AD sera. The mean titre of anti-wheat IgG was 6710±4520 units/ml in AD sera, and 1390±.807 units/ml in normal control sera. These findings indicated that atopic patients allergic to wheat proteins who had high levels of IgE antibody also had high levels of IgG antibody. Moreover, low levels of IgG antibodies were detected in normal controls, and there were persons who had relatively high titres of IgG antibody, suggesting that there were persons who had elevated IgG immune responses to wheat antigens whether or not they had IgE antibody. These results indicated that wheat proteins might contain several components or epitopes which could trigger an immune response in most humans, but disease-specific protein components or epitopes might exist which would result in IgE-mediated atopic dermatitis.

To determine the possible polypeptides that elicit disease-specific immune response, our study was extended to include the immunoblotting analysis. The solubilized polypeptides of wheat flour proteins were transferred electrophoretically to PVDF membrane that was cut into strips and incubated with sera from different patients.

IgG antibodies reacted mainly with polypeptides of molecular weight between 40 and 50 kDa, which were the major protein components of wheat. The IgA response was essen-

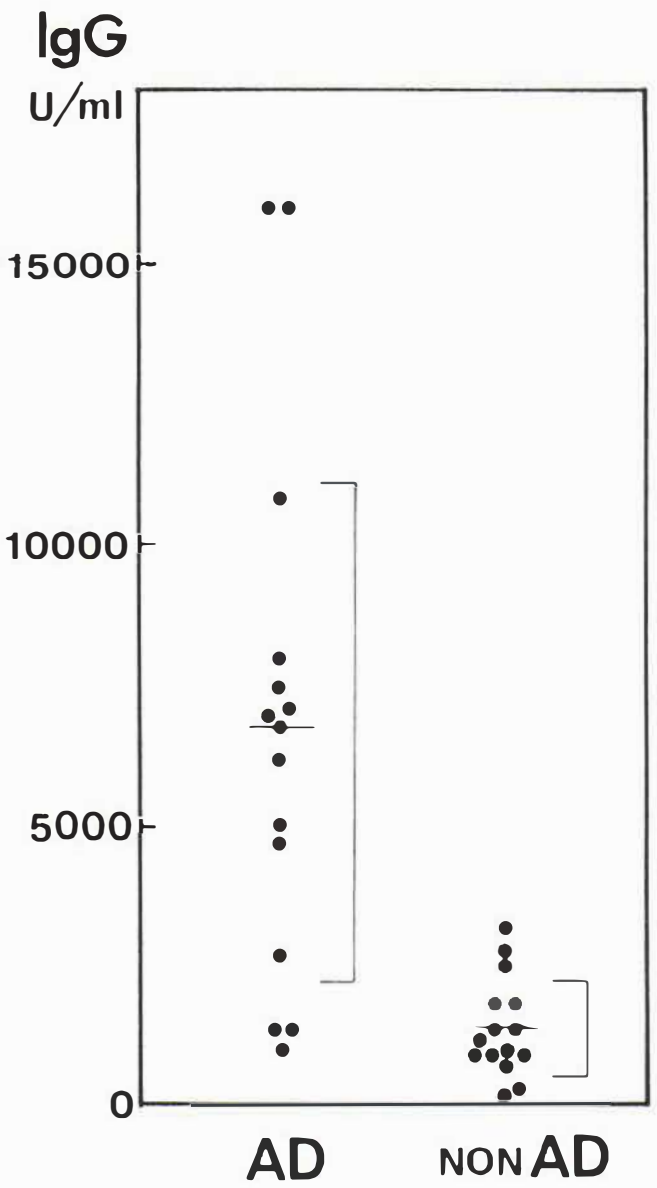

Fig. 3. Serum levels of IgG antibodies to wheat proteins in normal (*Non-AD*) and AD patients (*AD*) by ELISA methods. (IgG mean titres: *AD*: 6710±4520 units/ml; *Non-AD*: 1390±807 units/ml.

tially the same as the IgG response. Sera of AD patients were subjected to immunoblotting analysis. IgG antibodies in patient' sera recognized many polypeptides of molecular weight between 14 and 98 kDa and the density of each band was much stronger than that in normal sera. In particular, the polypeptides of molecular weight 16, 45, 55, and 60 kDa were found in much greater quantity than the others. Interestingly, in AD sera, additional bands could be detected at the molecular weights 14, 24, 29, and 70 kDa (Fig. 4).

IgA immunoblots revealed that essentially no differences could be detected between patients' sera and normal sera. No disease-specific IgA bands were found (Fig. 4).

The number of IgE antibodies which reacted to wheat proteins were much less than that of IgG and IgA antibodies. Sera from different patients appeared to have similar (but not identical) spectra of IgE antibodies to the individual wheat polypeptides. Since normal control sera showed no binding of IgE antibodies at all, these polypeptides of molecular weights 33, 42, 45, 55, 80, and 98 kDa were definitively disease-specific (Fig. 4).

DISCUSSION

This is the first report, as far as we know, that wheat flour contains allergenic proteins that can be extracted under mild condition.

The immune response to wheat flour proteins was elicited in both normals and AD patients, but statistically increased levels of wheat-specific IgG antibodies were detected in sera of AD patients, as shown by ELISA methods. The recognition patterns of IgG, IgA, and IgE-antibodies in normal sera and patients' sera were found to be variable by the immunoblotting technique, and the patients' sera contained IgG antibodies which react to many polypeptides in the wheat protein preparations. The IgG antibodies to the polypeptides of molecular weights 14, 24, 29, and 70 kDa were demonstrated in a disease-specific manner, because no IgG bands of these polypeptides were detected in normal sera tested.

IgE antibodies were also demonstrable. No IgE antibodies were found in normal sera at all, whereas IgE antibodies to wheat polypeptides of molecular weights, 33, 42, 45, 55, 80, and 98 kDa were detected solely in AD sera. Thus, hypersen-

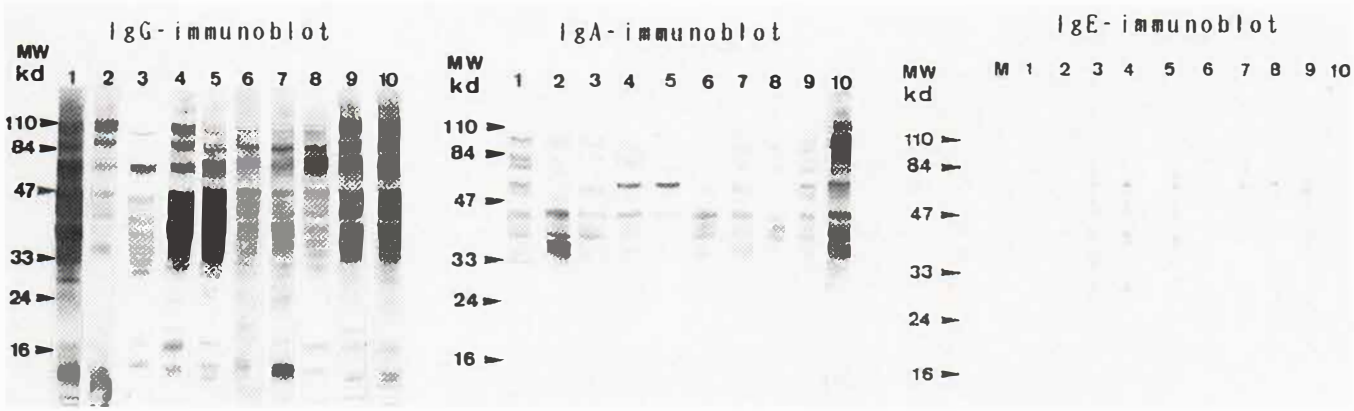

Fig. 4. IgG, IgA, and IgE immunoblots using sera from 10 patients with AD.

sitivity to wheat proteins can be demonstrated in wheat-IgE-RAST-positive sera of atopic dermatitis.

One of the interesting issues regarding atopic dermatitis and food allergy is whether the allergenic polypeptides are capable of inducing both IgG and IgE antibodies with the same specificity. Preliminary data using salt-extracted wheat proteins indicated that some of the IgG and IgE antibodies recognize the same molecular weight wheat polypeptides. We are also trying to remove the allergenic polypeptides from wheat flour to make hypoallergic wheat.