

Original article

Comparison of antibody responses to hen's egg and cow's milk proteins in orally sensitized rats and food-allergic patients

Background: No adequate enteral sensitization models are available to study food allergy and the allergenicity of food proteins. To further validate an enteral brown Norway (BN) rat sensitization model under development, we studied specific protein recognition to determine whether a comparable pattern of proteins is recognized by the rat immune system and the human immune system.

Methods: The animals were exposed to either ovalbumin as a positive reference control, hen's egg-white-protein extract, or a cow's milk preparation by daily gavage dosing (0.5, 1, 2.5, 5, 10, or 15 mg protein per rat/day) for 9 weeks. No adjuvants were used during the sensitization studies. The specificities of antibodies against hen's egg-white proteins or cow's-milk proteins in sera from orally sensitized rats and food-allergic patients were studied and compared by immunoblotting.

Results: The IgG and IgE antibodies to hen's egg-white proteins and cow's-milk proteins present in sera from orally sensitized rats and food-allergic patients showed a comparable pattern of protein recognition.

Conclusions: Upon daily intragastric exposure to food allergens, the specificities of the induced antibody responses in the BN rat resemble those found in food-allergic patients. These studies add further support to the hypothesis that the BN rat may provide a suitable animal model for food allergy research and research on the allergenicity of food proteins.

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Type I or IgE-mediated allergic reactions are known to play a major and primary role in food allergy (1). In genetically predisposed (atopic) patients, food allergy is generally caused by an overproduction of IgE against common dietary proteins (2). Food allergy is a problem of growing interest. This is so, firstly, because there are indications that an increasing number of sensitized individuals suffer from the clinical features upon a renewed contact with the offending food. Secondly, it is also anticipated that as a result of the rapid developments in biotechnology, which generally will result in the introduction of new proteins and new biological varieties for use as food, the potential allergenicity will pose a major problem in their safety assessment. The assessment strategy currently used seems to be promising for the testing of foods containing newly introduced gene products derived from common or less common allergenic sources. However, the possible transfer of a gene coding for an allergen from a source with no history of allergenicity is extremely difficult to predict

with the current available tools. Therefore, validated animal models to study food allergy and the allergenicity of food proteins are urgently needed. Unfortunately, no validated oral animal models to study food allergy and the allergenicity of food proteins are available yet.

We have previously reported an oral sensitization protocol to sensitize brown Norway (BN) rats to food proteins without the use of an adjuvant (3). In later studies, we confirmed that, by this sensitization protocol, the BN rat was the most suitable strain for inducing specific IgE responses when compared to Wistar, hooded Lister, and PVG rats, which all were found not to produce measurable levels of antigen-specific IgE in the applied enteral induction protocol (4). Results from other studies (5, 6) also indicate that the BN rat is the most suitable strain for oral sensitization studies. In addition, after an oral challenge in previously sensitized rats, local and systemic immune-mediated effects were demonstrated. In most

animals, an increased gastrointestinal permeability was demonstrated, and some animals showed a drop in blood pressure or breathing frequency, an effect which is in accordance with observations from human clinical practice (7). In the present paper, to further characterize our model, we studied specific protein recognition to determine whether a comparable spectrum of proteins is recognized by the rat and human immune systems. We exposed BN rats to a total hen's egg-white (HEW) protein extract and cow's milk (CM), and compared the specificities of induced antibody responses with the specificities of antibodies in sera from egg- and milk-allergic patients.

Since it is known that the dose of antigen influences the outcome of oral sensitization studies (8, 9), we used different doses of the antigens to sensitize the animals by daily gavage dosing. To investigate whether the test animals were sensitized to the different antigen sources and to determine which dose of antigen induced the best response, we determined antigen-specific antibodies by enzyme-linked immunosorbent assay (ELISA) and passive cutaneous anaphylaxis (PCA) tests. The sera with the highest levels of specific antibodies were subsequently used in immunoblotting to study specific protein recognition and to compare the binding patterns with those of antibodies in sera from allergic patients.

Material and methods

Animals and maintenance

Young male BN rats were obtained from Charles River (Sulzfeld, Germany). The rats were housed in an animal room maintained at $23 \pm 3^\circ\text{C}$, with a light/dark cycle of 12 h, and a relative humidity of 30–70% during the experiment and for at least 10 days prior to study initiation. The animals were housed in stainless-steel wire cages in groups of four and had free access to food and tap water. The rats were bred and raised on a commercially available hen's-egg-protein- and CM-protein-free rodent diet (SDS Special Diet Service, LAD1 (E) SQC, Witham, UK). Prestudy blood samples were always tested for specific antibodies to hen's-egg protein and CM protein to ensure the use of immunologically naive animals with respect to the antigens under investigation. All animal studies were approved by an independent ethics committee.

Materials

In these studies, three different antigen sources were used. A total HEW protein extract was prepared essentially by the method of Bernhisel-Broadbent et al. (10). Hen's eggs (free-range) were obtained from Albert Heijn, Zaandam, The Netherlands. A sample of 30 g of fresh egg white was added to 300 ml of sterile saline, and the mixture was rotated overnight at 4°C . The

mixture was divided over several tubes and subsequently centrifuged for 10 min at 2500 g and 4°C . The tubes were decanted and the supernatants were microcentrifuged for 15 min at 17000 g and 4°C and subsequently sterilized in passing through 0.2- μm filters (Costar Scientific Corporation, Cambridge, MA, USA). The protein content of the extract was determined with a BCA protein assay (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The total protein content of the extract was 24 mg protein/ml saline, and the total protein yield was approximately 6.5 g. The two other antigen sources used were ovalbumin (OVA) (Fluka Chemie, Buchs, Switzerland, purity: 70%) and skimmed CM (Albert Heijn, Zaandam, The Netherlands, 4 g protein/100 ml milk).

In the studies presented in this paper, we used sera of 38 young patients, aged from 7 months to 8 years, with previously proven IgE-mediated allergy to HEW (18 patients) or CM (18 patients), as determined by a positive family history for atopic disease, a positive radioallergosorbent test (RAST) (mean class was 1.8 ± 1.17), and positive skin prick test ($\text{SPT} \geq 2+$; data not shown).

Experimental design

Animals, 4–6 weeks old at study initiation, were exposed to either OVA, HEW proteins, or CM proteins by gavage dosing. Gavage dosing was performed daily for 9 weeks, using an 18-gauge, stainless-steel, animal-feeding needle. Groups of six BN rats were dosed orally with six different concentrations of one of the antigen sources (0.5, 1, 2.5, 5, 10, and 15 mg protein in 1 ml of tap water per animal per day). Blood samples were obtained from the orbital plexus under light CO_2 anesthesia at weekly intervals or by exsanguination from the abdominal aorta at death. After coagulation for 1 h at room temperature, the blood samples were centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 min at 2000 g and 4°C to obtain sera. The sera were stored at -20°C until analyses for specific protein recognition by IgG and IgE antibodies against proteins in the HEW-protein extract and CM with SDS-PAGE and immunoblotting. In addition, anti-OVA specific IgG and IgE titers and anti-HEW protein- and anti-CM protein-specific IgG titers were determined by ELISA. The method used to determine the antigen-specific IgE titers (digoxigenin-mediated assay; Knippels et al. [3]) is not suitable to determine specific IgE levels quantitatively for mixtures of proteins *but only for a single protein*. Therefore, anti-HEW-protein and anti-CM-protein IgE titers were determined by PCA tests.

Positive control animals ($n=4$ per antigen source) were obtained by intraperitoneal injection with 0.5 ml of a 0.2-mg protein/ml OVA, HEW-protein-extract, or CM solution in sterile saline on days 0, 2, 4, 7, 9, and 11.

To potentiate the immune response, 0.2 ml of a 25 mg/ml alum adjuvant suspension in sterile saline mixed with 0.5 ml of the OVA, HEW-protein-extract, or CM solution was injected on day 0. The animals were bled on day 28 by exsanguination from the abdominal aorta. Sera were prepared and stored as described above. These sera were pooled and used as positive control samples in the OVA-, HEW-protein-, CM-protein-specific ELISA and in the PCA tests.

To investigate whether the test animals were sensitized to the different antigen sources and to determine which dose of antigen induced the best response, antigen-specific antibodies were determined by ELISA and PCA tests. The sera with the highest levels of specific antibodies were subsequently used in SDS-PAGE and immunoblotting to study specific protein recognition and to compare the binding pattern, with specific protein recognition of antibodies in sera of allergic patients.

ELISA for specific antibodies

Serum antibodies specific for OVA, HEW proteins, or CM proteins were measured by ELISA. Antigen-specific IgG ELISA was performed essentially as described previously (3) with the exception of the coating, which was performed with 100 µl/well of a 5 µg/ml solution of OVA or CM proteins or with a 75-µg/ml solution of HEW proteins in carbonate buffer, pH 9.6. OVA-specific IgE ELISA was performed as described previously (3). To determine the antibody titer of the test sera, a prestudy serum pool was used as negative control. The pooled prestudy serum was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated by starting at a 1:4 dilution, and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All analyses were performed in duplicate. The serum pool derived from the intraperitoneally immunized animals was used as a positive control sample. Positive and negative control samples were incorporated for each 96-well plate.

PCA tests

PCA was tested essentially as described previously (3). Naive (untreated) BN rats were shaven on the back and flanks and injected intradermally with 0.1 ml of the test sera in serial dilutions, followed 64 h later with an intravenous injection of 1 ml of a 1:1 mixture of a solution of OVA, HEW proteins, or CM proteins (5 mg protein/ml in sterile saline) and a solution of Evans blue (2% in sterile saline). After 20–30 min, the animals were examined for positive responses. The diameter of dye extravasation at the site of the serum injection was

measured. The reaginic titer was read as the reciprocal of the furthest dilution giving a colored spot of at least 5 mm in diameter. Positive and negative control sera as used in the ELISA were assayed simultaneously with the test serum of each animal used for the PCA tests.

SDS-PAGE electrophoresis and immunoblotting

SDS-PAGE was performed essentially according to Laemmli (11) with 12% Tris-HCl polyacrylamide gels. Before loading, protein samples were diluted (skimmed CM proteins 1:80, HEW proteins 1:40) in 63 mM Tris-HCl, 2% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 1% (w/v) dithiothreitol, pH 6.8, and boiled for 10 min. A rainbow marker (Amersham International, UK) with molecular masses of 200, 97, 69, 46, 30, 21, and 14 kDa was used as reference. Electrophoresis was performed for 15 min at 80 V followed by 1 h at 160 V. If not used for immunoblotting, gels were stained with Coomassie brilliant blue R-250.

After SDS-PAGE, the separated proteins were transferred to a polyvinylidenedifluoride membrane (PVDF; Immobilon-P Transfer membrane; Millipore Corporation, Bedford, MA, USA) by a semidry electrophoretic apparatus, as described by Towbin et al. (12). The membranes were blocked with 3% (w/v) nonfat dry milk (Protifar; Nutricia, Zoetermeer, The Netherlands) or with 3% (w/v) BSA in 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl when electrophoresis was performed with HEW proteins or CM proteins, respectively. After 1.5-h blocking at room temperature, human or rat sera, diluted 1:5 in washing solution (0.1% Tween 20, 1% nonfat dry milk, or BSA) were added and incubated overnight at room temperature. After washing, the membranes were incubated for 3 h at room temperature with peroxidase-conjugated antibodies (rabbit antihuman IgG, rabbit antihuman IgE, goat antirat IgG, or mouse antirat IgE, Zymed, San Francisco, CA, USA), diluted 1:500 in washing solution. Subsequently, the blots were developed for peroxide activity by chloronaftol/DAB staining for approximately 5–20 min.

Results

ELISA and PCA tests with rat sera

Prestudy blood samples were all negative for OVA, HEW protein, or CM protein specific antibodies. These sera were pooled and used as negative control in the ELISA and PCA tests. Specific IgG and IgE antibodies were demonstrated in the pooled day-28 sera from the positive control animals ($^2\log$ IgG titer ≥ 11 in the OVA-, HEW-protein-, and CM-protein-specific IgG ELISA and $^2\log$ IgE titer ≥ 11 in the OVA-specific IgE ELISA; data not shown). These serum pools were

subsequently used as positive controls in the ELISA and PCA tests.

The all enteral treatment regimen induced specific antibody responses in the rats. In OVA-exposed rats, the most pronounced antibody responses were observed in the animals exposed to 1 mg OVA/day. Maximum OVA-specific IgG titers in these animals were demonstrated at day 56 (Fig. 1A: $^2\log$ titer 11.2 ± 5.2 [mean $\pm$ SD]; range: 5–20), and maximum OVA-specific IgE responses at day 42 (Fig. 1B: $^2\log$ titer 6.8 ± 3.03 ; range: 4–11). Higher doses of OVA induced less pronounced specific antibody responses, and only 4/6 animals responded upon daily gavage dosing with 10 mg OVA/rat (Fig. 1). The optimal antibody responses in HEW-protein-exposed rats were detected in animals exposed to 10 mg HEW proteins by daily gavage dosing with maximum HEW-protein-specific IgG responses detectable at day 49 with 100% responders (Fig. 2: $^2\log$ titer 10.33 ± 4.97 ; range: 5–16). Rats exposed to lower doses of HEW protein by daily gavage dosing developed lower levels of specific

antibody responses, as shown in Fig. 2 for the animals exposed to 2.5 mg HEW protein per rat/day. The optimal antibody responses in CM-protein-exposed rats were detected in animals exposed to 10 mg CM proteins by daily gavage dosing, with maximum CM-protein-specific IgG responses detectable at day 56 with 100% responders (Fig. 3: $^2\log$ titer 4.8 ± 0.84 ; range: 4–6). Since we were not able to measure CM-protein- and HEW-protein-specific IgE responses by ELISA, re-agenic antibody responses to CM proteins and HEW proteins were determined by PCA tests. Undiluted day-63 sera of 20 HEW-protein-sensitized animals and all 34 CM-protein-sensitized animals were analyzed by the PCA test. Only two HEW-protein-sensitized animals and two CM-protein-sensitized animals showed a positive PCA reaction (data not shown).

SDS-PAGE and immunoblotting

The total Coomassie brilliant blue staining of the proteins from the HEW-protein extract and CM after SDS-PAGE is presented in Fig. 4.

HEW-protein- and CM-protein-specific immunoglobulin binding was examined by immunoblotting with both sera from orally sensitized rats and food-allergic patients. Fig. 5 shows the binding of IgG and IgE antibodies to HEW proteins on immunoblots after incubation with the sera of egg-allergic patients (Fig. 5A: specific IgG binding; 5C: specific IgE binding) or HEW-protein-sensitized rats (Fig. 5B: specific IgG binding; 5D: specific IgE binding). The specific protein binding by IgG antibodies from HEW-protein-sensitized rats showed a pattern comparable to the protein binding by IgG antibodies in the sera of egg-allergic patients. Both sensitized rats and allergic patients

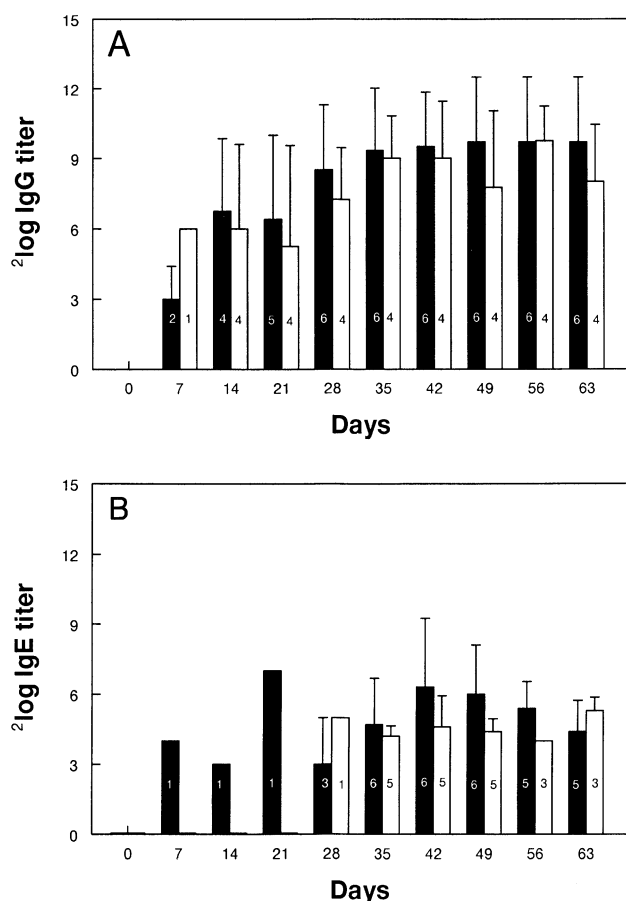


Figure 1. Time dependency of OVA-specific IgG (A) and IgE (B) response in young BN rats after daily intragastric dosing with 1 mg OVA per rat/day (black bars) or 10 mg OVA per rat/day (bright bars) for 63 days. Immunoglobulin titers were determined in blood samples obtained at weekly intervals. Data are presented as mean $^2\log$ Ig titer $\pm$ SD of six rats per group. Number of responders at respective time points is indicated in bars.

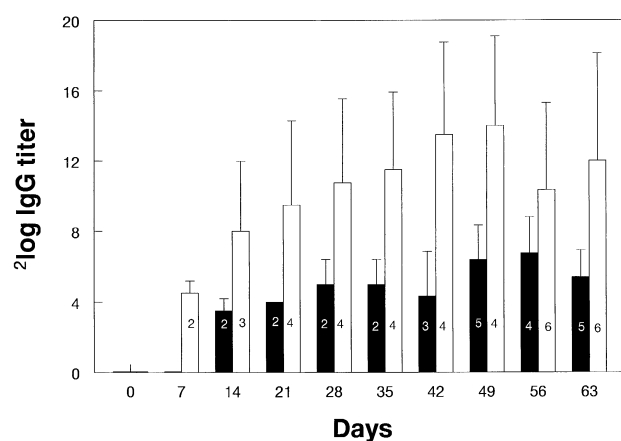


Figure 2. Time dependency of HEW-protein-specific IgG responses in young BN rats after daily intragastric dosing with 2.5 mg HEW protein per rat/day (black bars) or 10 mg HEW protein per rat/day (bright bars) for 63 days. Immunoglobulin titers were determined in blood samples obtained at weekly intervals. Data are presented as mean $^2\log$ IgG titer $\pm$ SD of six rats per group. Number of responders at respective time points is indicated in bars.

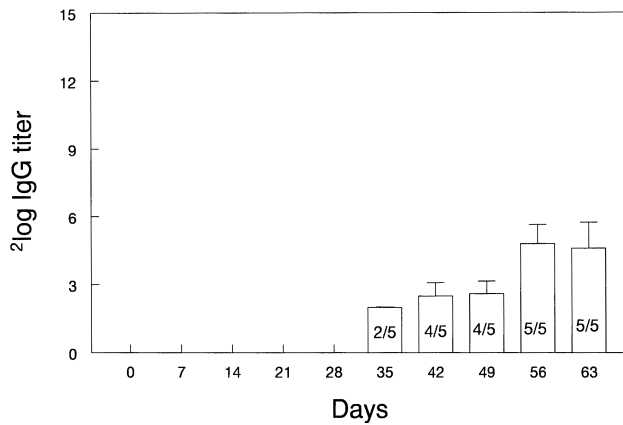


Figure 3. Time dependency of CM-protein-specific IgG responses in young BN rats after daily intragastric dosing with 10 mg CM protein per rat/day for 63 days. IgG titers were determined in blood samples obtained at weekly intervals. Data are presented as mean $2\log$ IgG titer $\pm$ SD of five rats per group. Number of responders at respective time points is indicated in bars.

mainly had IgG antibodies to ovotransferrin (77.7 kDa), OVA (46 kDa), ovomucoid (28 kDa), and lysozyme (14.3 kDa), although antibodies against other proteins were also observed. Although OVA and ovomucoid differ substantially in molecular mass, they migrate at very similar rates in SDS-PAGE, and are difficult to distinguish by this method (10, 13).

The resemblance in the IgE blots was even more striking. All patients tested produced specific IgE antibodies against ovotransferrin, ovomucoid, and OVA, whereas four patients demonstrated specific IgE antibodies against lysozyme. The same pattern was observed in specific IgE antibodies in HEW-protein-sensitized rats. All animals demonstrated specific IgE antibodies against ovotransferrin, ovomucoid, and OVA, whereas only five of the animals tested had produced detectable specific IgE antibodies against lysozyme. Fig. 6 shows the binding of IgG and IgE to CM proteins on immunoblots after incubation with sera of CM-allergic patients (Fig. 6A: specific IgG binding; 6C: specific IgE binding) or CM-sensitized rats (Fig. 6B: specific IgG binding; 6D: specific IgE binding). The specific protein binding by IgG antibodies from CM-sensitized rats resembled that by IgG antibodies in sera from CM-allergic patients. Both produced IgG antibodies to the main protein, β -lactoglobulin (18 kDa), and, to a lesser extent, to several caseins (around 30 kDa). All CM-allergic patients mainly had IgE antibodies against β -lactoglobulin, and some very weak reactions against the caseins were observed in a few patients. Although the response in the rat IgE blot was very weak, the observed IgE antibodies also seemed to be mainly directed against β -lactoglobulin. As shown in Fig. 4, both CM and the HEW-protein extract contained a large number of different proteins. The majority of proteins present in

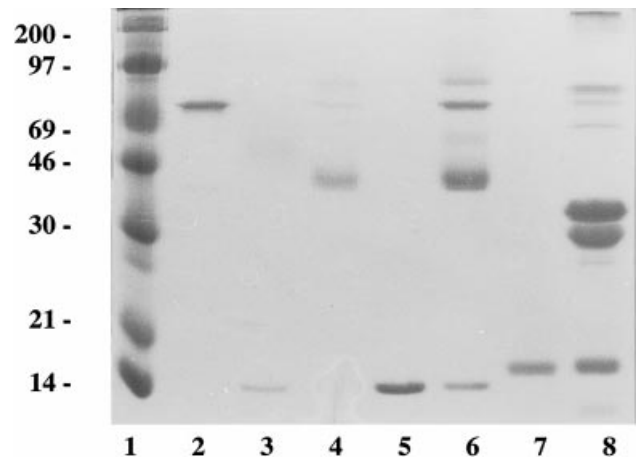


Figure 4. SDS-PAGE of proteins from CM, proteins from HEW extract, and purified proteins (Sigma Chemicals Co., St Louis, MO, USA). Lane 1) molecular weight marker, lane 2) ovotransferrin, lane 3) ovomucoid, lane 4) OVA, lane 5) lysozyme, lane 6) HEW extract, lane 7) β -lactoglobulin, lane 8) CM.

CM and the HEW-protein extract had not induced detectable IgE antibody responses in the BN rats, a fact which is in accordance with the observations of sera from the allergic patients.

Discussion

Using a previously reported oral sensitization protocol (3), we sensitized BN rats with different food allergens by daily gavage dosing. The allergen sources used were a total HEW protein extract and skimmed CM. Following the induction treatment, sera were collected for analysis of allergen-specific antibodies. The sera with the highest levels of specific antibodies against either HEW proteins or CM proteins, as determined by ELISA and PCA tests, were subsequently used in immunoblotting experiments to investigate whether the BN rat produced a profile of antibodies to HEW proteins and CM proteins similar to that observed in sera from food-allergic patients.

Immunoblotting experiments with rat sera demonstrated specific IgE antibodies against both HEW proteins and CM proteins, while only very few sera were positive in the PCA tests. These results suggest that immunoblotting experiments are more sensitive than PCA tests in determining specific IgE antibodies. Previously, we also demonstrated a higher sensitivity for specific IgE detection with the ELISA than the PCA test (3).

The profile of allergens recognized by the immune system of the BN rat appeared to be comparable to the profile of allergens recognized by allergic human patients. Specific IgG antibodies in sera from hen's egg-allergic patients and rats orally exposed to HEW proteins recognized a rather broad yet similar spectrum

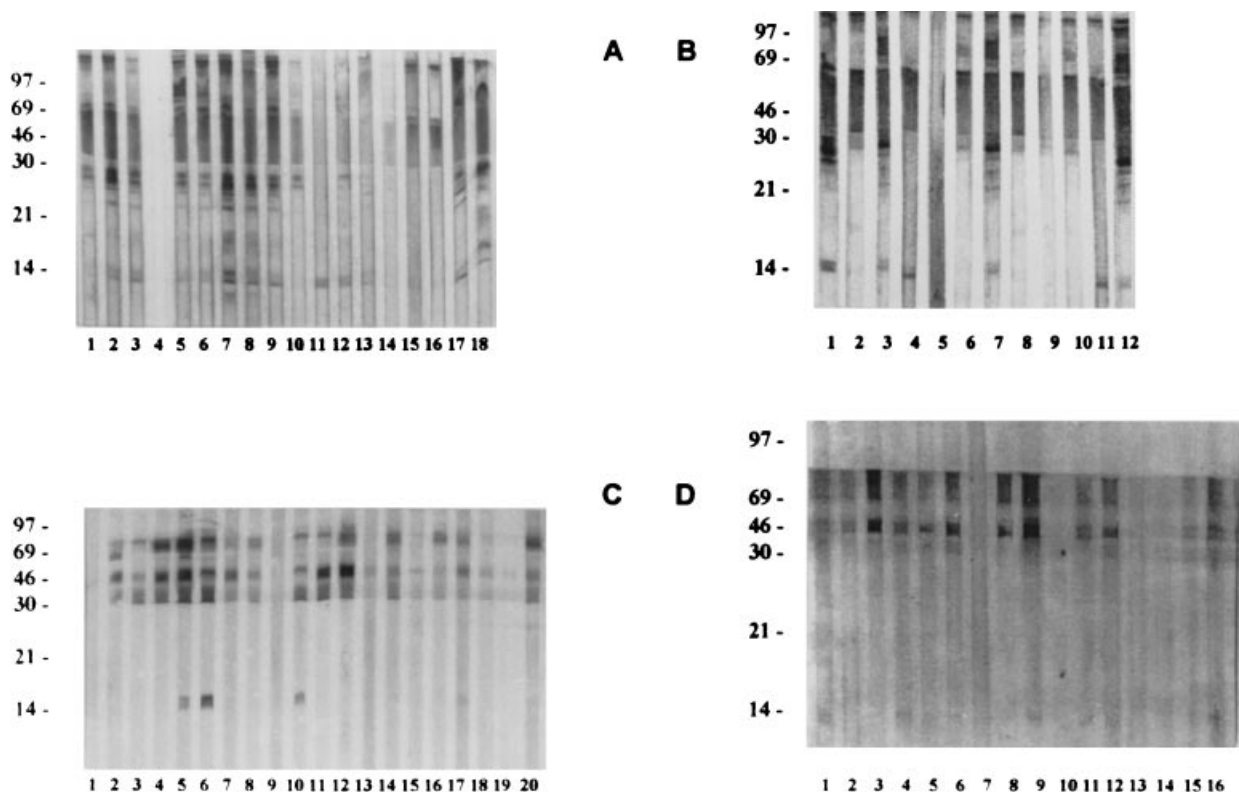


Figure 5. Immunoblot analysis of IgG and IgE antibodies against HEW proteins present in sera from hen's egg-allergic patients (blots A and C for IgG and IgE, respectively) and sensitized BN rats (blots B and D, respectively). Molecular masses are indicated on left in kDa. Blot A: Lanes 1–3, 5–13, and 15–18) sera from egg-allergic patients; lane 14) serum of nonallergic subject; lane 4) blank. Blot B: Lanes 1–4, and 6–12) sera from rats orally sensitized with HEW proteins; lane 5) serum from rat orally sensitized with CM proteins. Blot C: Lane 1) blank; lanes 2–8 and 10–20) sera from egg-allergic patients; lane 9) serum from nonallergic subject. Blot D: Lanes 1–6, 8, 9, and 11–16) sera from rats orally sensitized with HEW proteins; lane 7) blank; lane 10) serum from rat orally sensitized with CM proteins.

of proteins. Although egg white is a complex mixture of more than 20 proteins, particularly the specific IgE antibodies in sera from hen's egg-allergic patients and rats orally exposed to HEW proteins recognized the same proteins (mainly ovotransferrin, OVA, ovomucoid, and, to a lesser extent, lysozyme), and no reaction was observed against any other protein present in the HEW-protein extract. The same phenomenon was observed when the pattern of protein recognition by antibodies in sera from rats orally sensitized to CM proteins and antibodies present in sera from CM-allergic patients was compared. The induced antibodies were mainly directed against β -lactoglobulin and, to a lesser extent, against the caseins. Although CM contains more than 30 proteins (14), no reaction was observed against any other protein present in CM. These studies indicate that upon daily intragastric dosing with HEW proteins or CM proteins, the specific protein recognition of induced antibodies in the BN rat is comparable to that observed in sera from allergic patients. The same was described for BN rats intraperitoneally sensitized with CM, which produced a profile of IgE antibodies to milk proteins similar to that observed in man (5).

Our results obtained with sera from allergic patients are in accordance with human data in the literature since it is known that β -lactoglobulin is one of the most common allergens recognized by CM-allergic patients (15–19), although reactions are observed against other proteins from CM as well. In addition, ovotransferrin, OVA, ovomucoid, and lysozyme have been claimed to be the major allergens for hen's egg-allergic human patients (20–22). Although the induced antibodies in the BN rat apparently react to relevant proteins compared to the human situation, it remains to be elucidated whether the induced specific antibodies in the rat react to the same epitopes as the antibodies in the sera from patients. Since this is of critical importance, further studies will be conducted. Furthermore, it should be recognized that the observed differences in responses to the different food proteins as observed for the experimental animals as well as for patients are probably due to a combination of factors, such as the dose of allergen known to influence the outcome of oral sensitization (8, 9), and the allergenicity of the proteins used.

We have previously reported an oral sensitization protocol, without the use of an adjuvant, to sensitize

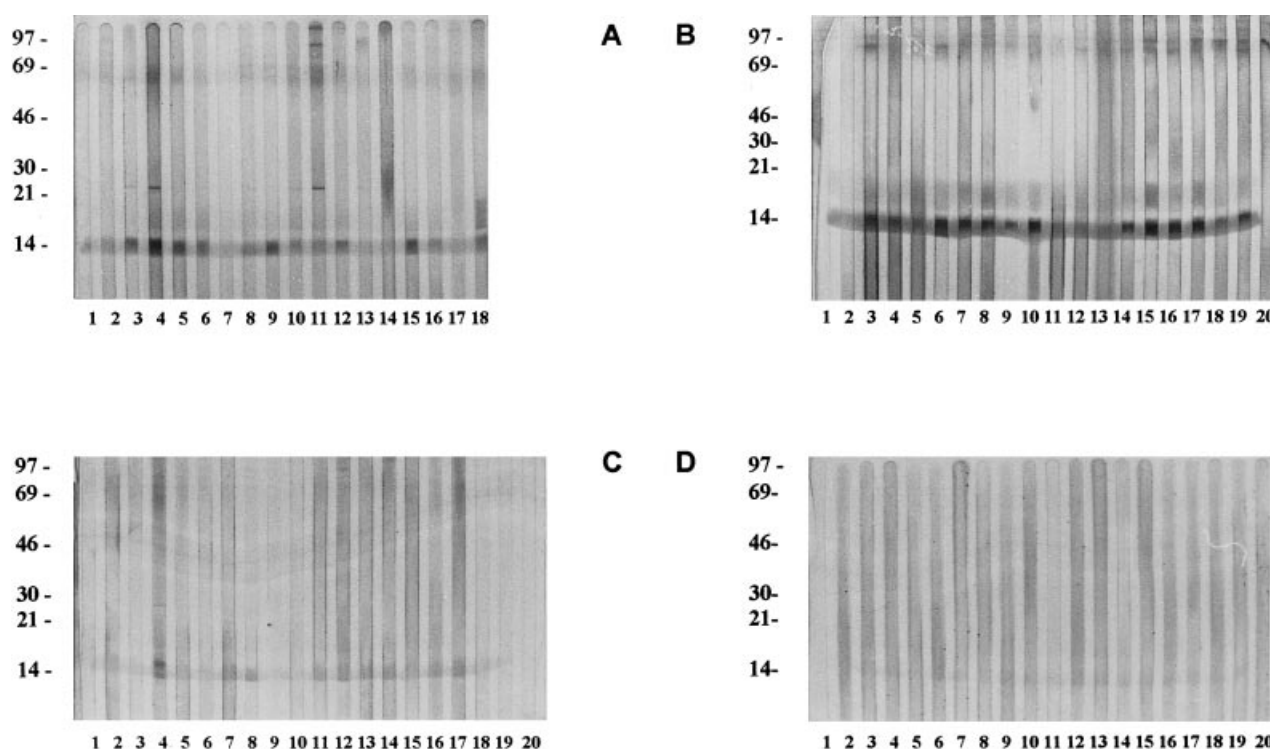


Figure 6. Immunoblot analysis of IgG and IgE antibodies against CM proteins present in sera from CM-allergic patients (blots A and C for IgG and IgE, respectively) and sensitized BN rats (blots B and D, respectively). Molecular masses are indicated on left in kDa. Blot A: Lanes 1–18) sera from CM-allergic patients. Blot B: Lane 1) blank; lanes 2–19) sera from rats orally sensitized with CM proteins; lane 20) serum from rat orally sensitized with HEW proteins. Blot C: Lane 1) blank; lanes 2–19) sera from CM-allergic patients; lane 20) serum from nonallergic subject. Blot D: Lane 1) blank; lanes 2–19) sera from rats orally sensitized with CM proteins; lane 20) serum from rat orally sensitized with HEW proteins.

BN rats to food allergens (3). In later studies, we confirmed that, by this sensitization protocol, the BN was the most suitable strain for inducing specific-IgE responses compared to Wistar, hooded Lister, and PVG rats, which all were found not to produce measurable levels of antigen-specific IgE in the applied enteral induction protocol (4). In addition, after an oral challenge in previously sensitized rats, local and systemic immune-mediated effects were demonstrated in accordance with observations from human clinical practice (7). In the present paper, to further validate our enteral food allergy model, we studied specific protein recognition to determine whether a comparable spectrum of proteins is recognized by the rat and human immune systems. The present studies indicate that enterally exposed BN rats and young patients demon-

strate IgE antibody responses to a comparable selection of proteins upon exposure to different protein mixtures, and add further support to the hypothesis that the BN rat may provide a suitable animal model for food allergy research and research on the allergenicity of food proteins.

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