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Effect of Antigen Dose on Induction of β-Lactoglobulin-specific IgG and IgE in Brown Norway Rats

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We established enzyme-linked immunosorbent assay (ELISA) which can detect 0.4−4 ng/ml of IgE by an avidin-biotin method, and 2-20 ng/ml of IgG or 5-50 ng/ml of IgM by direct ELISA methods. The ELISA methods were applied to measure serum immunoglobulin titers of Brown Norway rats intraperitoneally immunized with various amounts of β-lactoglobulin using an alum adjuvant. Induction of antigen-specific IgG was predominant at high antigen doses such as 100 and 1000 μg/rat, but markedly decreased at low antigen doses such as 1 and 10 μg/rat. Induction of antigen-specific IgE was not so affected by antigen dose, but was much weaker than IgG induction and was temporal.

INTRODUCTION

Recently, food allergy has become a serious health problem not only for children but also for adults. Some types of food allergy is mediated by allergen-specific IgE bound to mast cells or basophils. The binding of allergen to the bound IgE induces degranulation of these cells and release inflammatory mediators such as histamine, platelet activating factor and leukotriens. Although the serum IgE level of healthy individuals is very low, it often elevates markedly in allergy patients. Thus, the measurement of total and antigen-specific IgE is crucial for the diagnosis of food allergy.

To detect antigen specific IgE, radioallergosorbent test is used most frequently (Adler *et al.*, 1991; Diaz-Sanchez and Kemeny, 1990, 1991 ; Diaz-Sanchez et *al.*, 1993 ; Ogawa *et al.*, 1991) and passive cutaneous anaphylaxis (PCA) test is also available (Jarrett and Stewart, 1974, 1976 ; Bazin and Plateau, 1976 ; Sedgwick and Holt, 1983, 1985). Although these methods can detect IgE with high sensitivities, the former requires the facility for handling of radioactive materials and the latter does the animal experiment facility. Enzyme-linked immunosorbent assay (ELISA) (Engvall *et al.*, 1971) can be a substitute for the radioactive assay, and is used for a quantitative determination of various proteinous substances. Several researchers are using the ELISA system to measure antigen-specific IgE in human (Doke *et al.*, 1989 ; Wadee *et al.*, 1990), mouse (Fehler *et al.*, 1991) and rat (Fritsche and Bonzon, 1990 ; M. Yamada *et al.*, 1992) .

To clarify the mechanism of food allergy, establishment of animal model system is essential. Among experimental animals being used for immunological studies, Brown Norway (BN) rats produce higher amounts of IgE than other rat strains (Diaz-Sanchez and Kemeny, 1991). Fritsche and Bonzon (1990) reported the induction of antigen-specific IgE by immunizing BN rats with β-lactoglobulin (β-LG), estimating

serum IgE content by PCA and ELISA methods. We tried to reproduce the results, but found that their ELISA system detected IgG as well as IgE. Thus, we newly established an ELISA method to detect rat IgE more specifically for analysis of serum IgE in BN rats immunized with β-LG.

MATERIALS AND METHODS

Materials

β-LG was purchased from Sigma Chemical Co. (St. Louis, MO). In ELISA experiments, 0.05% Tween 20 dissolved in a phosphate buffered saline, pH 7.4 (TPBS) was used for rinsing and 0.1% fish gelatin (Sigma) dissolved in TPBS was used for blocking (Fritsche and Bonzon, 1990). The blocking solution was used for dilution of antibodies used for ELISA. Control rat IgE and IgM were purchased from Zymed (San Francisco, CA) and IgG from Dainihon Pharmaceutical Co. (Osaka, Japan). Goat anti-rat IgE was purchased from Bethyl (Montgomery, TX), goat anti-rat IgM $(Fab')_2$ and its peroxidase (POD) conjugate from Cappel (West Chester, PA), rabbit anti-rat IgG $(Fab')_2$, its POD conjugate, and POD-conjugated rabbit anti-rat IgE from Zymed, biotin-conjugated mouse anti-rat IgE from Biosoft (Paris, France), POD-conjugated avidin from Dakopatts (Glostrup, Denmark). Substrate solution for the ELISA was a 10 : 9 : 1 mixture of 0.006% H_2O_2 dissolved is 0.2M citrate buffer (pH 4.0), H_2O, and 6 mg/ml of 2,2'-azinobis (3-ethylbenz-thiazoline sulfonic acid). Two types of 96 well immunoplates, Maxisorp and Polysorp (Nunc, Roskilde, Denmark) were used for the ELISA

Immunization

Male BN/Sea [SPF] rats (4 weeks old) were intraperitoneally immunized 3 or 4 times with β-LG dissolved in 3% Al $(OH)_3$ − 0.15M NaCl and blood sample was collected from their tails at intervals. Finally, rats were anesthetized with ether and blood was collected from aorta. Then, serum was prepared by centrifuging the blood at 3000Xg for 15 min.

ELISA

Measurements of total immunoglobulins were executed using sandwich ELISA methods. Goat anti-rat IgE, rabbit anti-rat IgG $(Fab')_2$, or goat anti-rat IgM $(Fab')_2$ was used to fix each immunoglobulin. These antibodies were diluted 1,000 times with 50 mM sodium carbonate-sodium bicarbonate buffer (pH 9.6) and 96 well plates were treated with 100 μl of the solution for 1 hr at 37°C. After blocking with 300 μl of the blocking solution for 1 hr at 37°C, each well was reacted with 100 μl of standard immunoglobulin solutions dissolved in the blocking solution or rat serum diluted with the same solution for 1 hr at 37°C. Then, bound IgG was detected by reacting with 100 μl of POD-conjugated rabbit anti-rat IgG $(Fab')_2$ (2000 times diluted), IgM with 100 μl of POD-conjugated goat anti-rat IgM $(Fab')_2$ (1000 times diluted). Bound IgE was detected by two methods ; directly reacting with 100 μl of POD-conjugated mouse anti-rat IgE (1000 times diluted), or reacting with biotin-conjugated mouse anti-rat IgE (1000 times diluted) and then with POD-conjugated avidin (5000 times diluted). Wells

were rinsed 3 times with TPBS between each step. After incubating at 37°C for 15 min with 100μl of substrate solution, the reaction was stopped by adding 100μl of 1.5% oxalic acid and absorbance at 415 nm was measured with an ELISA reader (Tohso Co., Tokyo, Japan). To measure antigenspecific antibody, 150μl of antigen solution (50 μg/ml) dissolved in the 50 mM carbonate-bicarbonate buffer (pH 9.6) was added to each well of 96 well ELISA plate, and then processed as above.

RESULTS

Establishment of ELISA for measurements of rat immunoglobulins.

Figure 1 shows calibration curves of rat IgM, IgG and IgE obtained by sandwich ELISA methods using two types of ELISA plates, Maxisorp and Polysorp. In the case of direct ELISA determinations for IgM and IgG, Maxisorp gave approximately 6 times higher sensitivity than Polysorp. The direct ELISA methods could detect 2-20 ng/ml of IgG and 5-50 ng/ml of IgM. In the case of IgE, the avidin-biotin method using biotinated anti-rat IgE and avidin-POD detected IgE with a sensitivity approximately 9 times higher than the direct ELISA assay using a POD-conjugate of mouse anti-rat IgE, and Maxisorp again gave much higher sensitivity than Polysorp. The avidin-diotin method using Maxisorp could detect 0.4-4 ng/ml of IgE.

Measurement of total and β-LG-specific antibodies in immunized rat.

BN rat was intraperitoneally immunized with 100 or 1000 μg or β-LG in the 1st

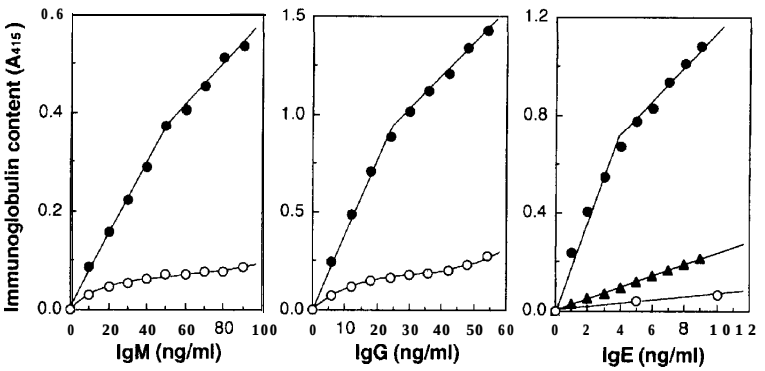

Fig. 1. Calibration Curves of Rat Serum IgE, IgG and IgM by the Sandwich ELISA Method. For IgG and IgM determinations, the direct ELISA method was employed using Polysorp (○) or Maxisorp (●) For IgE determination, the avidin-biotin method was employed using Polysorp (○) or Maxisorp (●), or the direct ELISA mathod using Maxisorp (▲).

immunization (day 0) and then with 50 μg in the 2nd (day 13) and 3rd immunizations (day 27) using an alum adjuvant to induce antigen-specific antibodies. The amounts of total and antigen-specific antibodies were measured by the ELISA methods described above. The sensitivity of the ELISA established here was high enough and these sera should be diluted 10^5-10^6 times for IgG, 10^3-10^4 times for IgM, and 10 times for IgE to determine their levels accurately. As shown in Fig. 2, total serum IgE levels were 52 $\pm$ 6 ng/ml in nonimmunized rats and 200 $\pm$ 45 ng/ml in the rats immunized with 1000 μg of the antigen ($-times increase) . Similarly, total IgG level increased from 2. 23f0.33 to 4.00 $\pm$ 0.40 mg/ml (1.8-times increase) and IgM from 0.37 $\pm$ 0.09 to 0.61 $\pm$ 0. 06 mg/ml (1.6-times increase) by the immunization.

In the case of antigen-specific antibodies, IgG was induced markedly, IgM weakly, and IgE slightly by the immunization (Fig. 3) . In nonimmunized rats, weak background coloring was observed in undiluted and 10 times diluted sera in IgM and IgG determinations. On the other hand, the background coloring was observed even in 10^3 times diluted sera in IgE determination using the avidin-biotin method. In immunized rats, significant increase of antigen-specific antibodies was observed in 10^2 times -diluted sera for IgM and 10^4 times-diluted sera for IgG, but the increase of specific IgE was marginal. The difference of specific IgE level between immunized and nonimmunized rats was the largest in 10^2 times-diluted sera. The samples obtained from the rats immunized with 100 μg of the antigen gave the similar result.

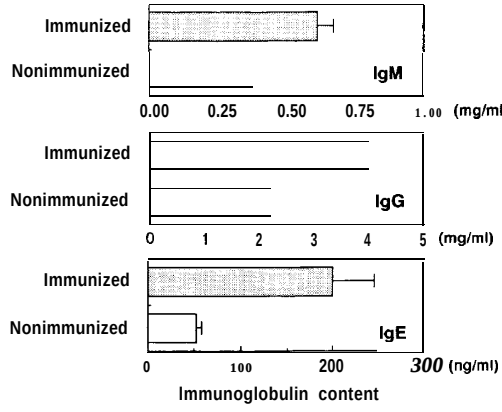

Fig. 2. Determination of Total Antibodies in Sera of Brown Norway Rats Immunized with β-Lactoglobulin. Serum immunoglobulin levels of three RN rats immunized with 1 mg/rat of β-LG at the 1st immunization (dotted bar) and those of three nonimmunized rats (open bar) were measured by the ELISA methods established here. Mean $\pm$SE was shown in each group.

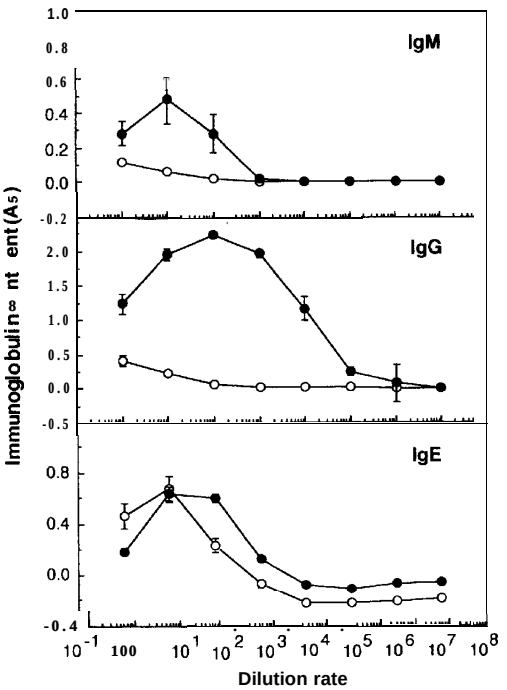

Fig. 3. Determination of β-Lactoglobulin-specific Antibodies in Sera of Immunized and Nonimmunized Brown Norway Rats. The amounts of antigen-specific antibodies of nonimmunized (0) and immunized (●) rats were measured by the ELISA methods. Mean±SE of 3 rats was shown in each group.

Courses of antigen-specific IgG and IgE.

Since the antigen doses applied above predominantly induced antigenspecific IgG, rats were immunized with smaller amounts of the antigen to reduce IgG production. In this experiment, rats were immunized with 1, 10, or 100 μg/rat of β-LG on day 0, and then 10 μg/rat on days **14, 27,** and 34. After blood was sampled on days 11, 25, 32, and 36, rats were killed on day 40.

As shown in Fig. 4, antigen-specific IgG was not induced on day 11 yet, but induced on day 25 in the rats immunized with 100μg of antigen. When the rats were immunized with 1 or 10μg of the antigen in the 1st immunization, the induction of specific IgG was delayed and the maximum IgG levels also decreased with the decrement of antigen dose. On the contrary, IgE production was not so affected by the antigen dose. The antigen-specific IgE was obviously induced on day 25, gave a peak value on day 32, and then decreased rapidly on day 36, irrespective of the 4th immunization

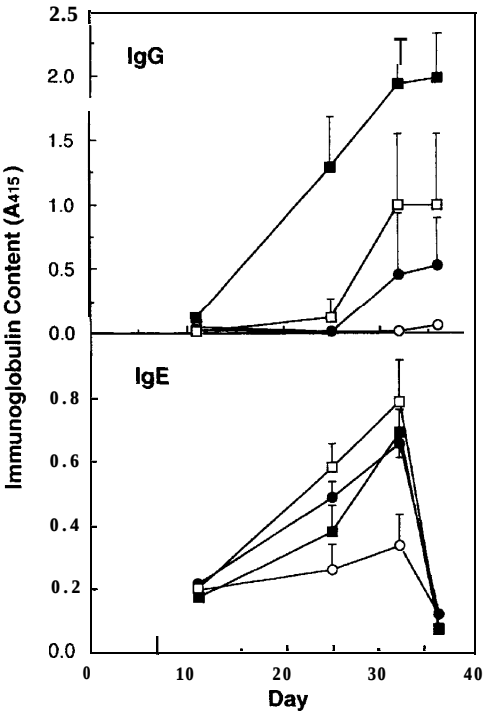

Fig. 4. Dose-dependent Induction of β-Lactoglobulin-specific IgG and IgE. BN rats were intraperitoneally immunized with 1 (●), **10** (□), or 100 (■) μg/rat of β-LG on day 0, and then boosted with 10μg/rat of the antigen on day 14, 27, and 34. Sera of nonimmunized (○) and immunized rats were prepared on day 11, 25, 32, and 36. The sera were diluted 100 times with blocking solution to measure the amounts of antigenspecific IgE and IgG. Mean ±SE of 3 rats was shown in each group.

on day 34. Such decrement of immunoglobulin level on day 36 was not observed in antigen-specific IgG.

DISCUSSION

To clarify the mechanism of food allergy, induction of allergen-specific IgE has been extensively studied using various rat strains. Though PCA reaction had been predominantly used for the detection of IgE (Jarrett and Stewart, 1974, 1976 ; Bazin and Platteau, 1976 ; Sedgwick and Holt, 1983, 1985), RAST (Diaz-Sanchez and

Kemeny, 1990, 1991) and ELISA (Fritsche and Bonzon, 1990 ; M. Yamada et *al.*, 1992) methods are using recently. Though ELISA is less sensitive than the others, it is easier to assay many samples quantitatively. Since an ELISA method reported for rat IgE determination (Fritsche and Bonzon, 1990) was not specific to IgE, we newly established a more specific and sensitive ELISA method for rat IgE. Though milk proteins such as bovine serum albumin and a bovine skim milk preparation are often used for blocking of nonspecific adsorption of antibodies (K. Yamada *et al.*, 1989, 1992; M. Yamada et al., 1992), these proteins are also food allergens to be studied. Thus, a low allergenic fish gelatin was used as blocking reagent, according to Fritsche and Bonzon (1990). Among two ELISA plates tested here, Maxisorp having polar surface gave higher sensitivities than less polar Polysorp, probably by higher adsorption of fixing antibody. Thus, Maxisorp was employed in the ELISA methods. The sensitivity of the avidin-biotin method established here (0.4-4 ng/ml) was much higher than the avidin-biotin method reported previously (10-800 ng/ml) (M. Yamada *et al.*, 1992) and was high enough for determination of total and antigen-specific antibodies.

When BN rats were intraperitonealy immunized with 100 or 1000μg/rat of β-LG using alum adjuvant, antigen-specific IgG was strongly induced, whereas the induction of IgE was much weaker. Since the simultaneous presence of huge amounts of specific IgG inhibits the determination of specific IgE (Lee *et al.*, 1988) , antigen dose was then reduced from 100 to 1μg/rat to decrease IgG expression. The experiment showed that induction of antigen-specific IgG was highly dose dependent. On the other hand, the IgE induction was not so affected by antigen dose, as reported by Jarrett and Stewart (1974) . This means that smaller amounts of antigen below 10μg/rat is more appropriate to detect induction of IgE.

Though the ELISA system entablished here had enough sensitivity to detect serum IgE of BN rats, the induction of IgE was very weak and short-lived. After giving a peak value on day 32, IgE level of immunized rat decreased rapidly on day 36, irrespective of 4th immunization injected 2 days before sampling. Such temporal increase of specific IgE level has also been reported (Jarrett and Stewart, 1974 ; Meacock and Marsden, 1976). These results suggest that IgE production is subjected to negative regulation in *vivo*. In fact, Diaz-Sanchez and Kemeny reported the induction of high and long-lived IgE production in BN rats by co-administration of ricin (Diaz-Sanchez and Kemeny, 1991), which depress suppressor T cell activity (Diaz-Sanchez and Kemeny, 1990 ; Diaz-Sanchez et *al.*, 1993). These results suggest that some disorder in the suppressing system may lead to IgE spoduction. Thus, studies on the factors affecting the IgE production suppressing system should be important for prevention and remission of food allergy.

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