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# Identification of IgE with low molecular weight from serum of rats sensitized with DNP-Ascaris

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## ABSTRACT

Rat immunoglobulin E (IgE) was purified from the serum of Wistar rats sensitized with DNP-Ascaris. The IgE purification was carried out by precipitation with 50% saturated ammonium sulfate, ion-exchange chromatography on DEAE-Sephacel, affinity chromatography on goat anti-rat IgG (Fc)-Sepharose 4B and Protein A-Sepharose CL-4B, and gel filtration on Sephadex G-150. The results of the passive cutaneous anaphylaxis (PCA) reaction indicated that the protein fractionated by gel filtration on Sephadex G-150 was a rat IgE, and its molecular weight was approximately 130,000 on SDS-PAGE analysis. The rat IgE obtained in the present study was designated as low molecular weight (LMW) rat IgE. The molecular weight of the LMW rat IgE corresponded to that of the myeloma IgE fragment (LMW rat myeloma IgE) produced by digestion of rat myeloma IgE (IR 162) with papain. The LMW rat IgE did not react with peroxidase (POD)-goat anti-rat myeloma IgE (Fc) on Western blotting. Conversely, LMW rat myeloma IgE reacted with POD-goat anti-rat myeloma IgE (Fc) on Western blotting. These findings indicate that structural differences between LMW rat IgE and LMW rat myeloma IgE may exist in the Fc region. *Ryukyu Med. J., 16(3)111~116, 1996*

Key words: rat, IgE, passive cutaneous anaphylaxis (PCA) reaction, anti-rat myeloma IgE (Fc)

## INTRODUCTION

Immunoglobulin E (IgE) is the least component of the immunoglobulins in humans and experimental animals. Myeloma IgE proteins secreted from immunocytomas of LOU/Wsl rats[1] are therefore available for research on the structure, physicochemical properties and biologic activities of IgE[2~4]. Rat myeloma IgE proteins are, however, derived from IgE-producing myelomas, and are not antigen-specific IgE antibodies produced in the process of allergic reactions. It is considered therefore that an antigen-specific rat IgE antibody facilitates animal experiments on pathophysiological investigations of IgE-mediated allergic reactions. On the other hand, some investigators have tried to purify polyclonal IgE from the serum of rats infected with the nematode, *Nippostrongylus brasiliensis*[5], serum of atopic patients[6], and serum of bovine stimulated with rabbit serum albumin[7]. Although they succeeded to enrich IgE and to identify the protein, polyclonal IgE has never been purified. It is considered that the difficulty in purification of IgE is attributed to that IgE is the least component of the immunoglobulins and that various proteases which may degrade IgE proteins exist in the serum. It is expected therefore that digestion of IgE with proteases may give rise to IgE with low molecular weight in the circulating blood. In the present study, rat IgE was purified from the serum of Wistar rats sensitized with DNP-Ascaris (As). The immunochemical properties of the rat IgE obtained were compared with those of rat myeloma IgE (IR 162) digested with papain.

## MATERIALS AND METHODS

*Animals*

Male Wistar rats weighing 200~250 g were purchased from Kyudo Co., Ltd. (Kumamoto, Japan). We followed the "Standards Relating to the Care and Management of Experimental Animals" (Notification No.6, March 27, 1980, from the Prime Minister's Office, Tokyo, Japan) for the care and use of the animals, and the guide for animal experiments of the University of the Ryukyus. All animal studies were reviewed and approved by the Animal Care Committee at the University of the Ryukyus. The rats received normal non-purified diet (CLEA JAPAN, INC., Osaka, Japan) throughout the experimental period. This standard rat diet contains 25.5% protein and 4.3% fat by

weight.

*Reagents*

Following reagents were used; 2,4-dinitrobenzenesulfonic acid sodium salt (DNBS) (Tokyo Kasei Ind. Co. Ltd. Tokyo, Japan), killed *Bordetella pertussis* (Kaken Pharmaceut. Co. Ltd. Tokyo, Japan), mercuripapain and iodoacetic acid (Sigma Chemical Co. St. Louis, MO, U.S.A.), 3,3'-diaminobenzidine tetrahydrochloride, Tris (hydroxymethyl)-aminomethane and agarose-LGT (Nacalai Tesque, Inc. Kyoto, Japan), hydrogen peroxide (Santoku Chemical Ind. Co., Ltd. Miyagi, Japan), goat anti-rat IgG (Fc) (Chemicon International Inc. Temecula, CA, U.S.A.), peroxidase (POD)-conjugated goat anti-rat myeloma IgE (Fc) (Nordic Immunological Laboratories, Tilburg, Netherlands), rat myeloma IgE (IR 162) (Zymed Laboratories, Inc. San Francisco, CA, U.S.A.), sheep antisera to rat immunoglobulins ($IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_{2c}$, IgM and IgA) (The Binding Site Ltd. Birmingham, UK), and DEAE-Sephacel, Protein A-Sepharose CL-4B, CNBr-activated Sepharose 4 B, Sephadex G-150, Phast gel (gradient 10-15) and Phast gel sodium dodecyl sulfate (SDS) buffer strips (Pharmacia LKB, Biotechnology AB Uppsala, Sweden). Unless otherwise stated, all other chemicals were reagent grade.

*Conjugation of dinitrophenyl (DNP) groups to Ascaris extracts (As)*

Live *Ascaris suum* was obtained from the intestinal tract of pigs at the Okinawa Meat Hygiene Inspection Center (Okinawa, Japan). Proteins were extracted from the *Ascaris suum* by the method of Strejan & Campbell[8]. The extracted proteins were conjugated with DNP groups following the procedures of Eisen et al.[9].

*Sensitization*

Sensitization of the rats was carried out according to the method of Tada & Okumura[10]. One milligram of DNP-As suspended in 0.5 ml of saline was mixed with killed *Bordetella pertussis* (Bp) ($10^{10}$ cells). This mixture was aliquoted into 4 solutions and was injected subcutaneously into the footpads of the rats. Five days after the first injection, the rats were boosted with 0.5 mg of DNP-As suspended in 0.25 ml of physiological saline. One hundred Wistar rats sensitized with DNP-As were killed and bled between day 8 and day 15. After the blood had been allowed to clot at 4℃ overnight, 500 ml of the sera were pooled and stored at −70℃.

*PCA reaction*

Estimation of the passive cutaneous anaphylaxis (PCA) titer was performed according to the method of Tada & Okumura[10]. Fifty microliters of test sample was injected intradermally into the back of a non-treated Wistar rat. Forty eight hours later, 0.5 ml of 1% Evans blue containing 2 mg/ml of DNP-As was injected intravenously. Thirty minutes after the administration of Evans blue, the Wistar rat was killed and the blue spot on the skin was measured. A test sample yielding a blue spot in excess of 5 mm in diameter was regarded as positive for the PCA reaction.

*Ouchterlony double diffusion test*

Agarose-low gelling temperature (LGT) (1.2%) was dissolved in Veronal buffer, pH 8.6. After the agarose had been boiled and so become fully dissolved, the materials were placed on a glass plate. The mixture solidified perfectly and a small well (2 mm in diameter) was made in the gel. Then, 7$\mu$l of sample was applied to the well in the agarose gel. The agarose plate was allowed to stand for 48 h at room temperature, and the precipitin band that developed was stained with Coomassie blue after washing.

*Immunoadsorbent*

Cyanogen-bromide (CNBr)-activated Sepharose 4B was utilized for the preparation of goat anti-rat IgG (Fc)-Sepharose 4B according to the manufacturer's instructions.

*Purification of IgE from serum of rats sensitized with DNP-As*

The procedures for the purification of rat IgE were carried out as follows. At each purification step, the IgE contained in the materials was detected by the PCA reaction, and any other class of contaminant immunoglobulin in the materials was analyzed by the Ouchterlony test. Serum of the Wistar rats sensitized with DNP-As was precipitated with ammonium sulfate at 50% saturation at 4℃, redissolved in PBS, and extensively dialyzed against PBS. The materials containing rat IgE were further purified by ion-exchange chromatography on DEAE-Sephacel. The adsorbed proteins were eluted with a linear gradient of 0.05 M Tris-HCl, pH 8.1, to 0.05 M Tris-HCl, pH 8.1, 0.8 M NaCl. The proteins from the fractions which displayed PCA activities were further purified by affinity chromatography on goat anti-rat IgG (Fc)-Sepharose 4B, affinity chromatography on Protein A-Spharose CL-4B and gel filtration on Sephadex G-150.

*Digestion of rat myeloma IgE (IR 162) with papain*

Digestion of rat myeloma IgE (IR 162) with papain was performed according to the method of Rousseaux-Prévost et al.[4]. Myeloma IgE IR 162 (2 mg/ml) in PBS, pH 7.4, containing 2 mM ethylenediaminetetraacetic acid disodium salt (EDTA) was incubated at 37℃ for 10 min with mercuripapain using an enzyme to protein ratio of 1/100. The digestion was stopped by the addition of iodoacetic acid (final concentration: 15 mM).

*SDS-PAGE*

Electrophoresis was carried out according to the separation procedure used in a Phast System. The sample was incubated for 30 min at room temperature in 10 mM Tris-HCl (pH 8.0) containing 2.5% SDS, 1 mM EDTA. Polyacrylamide gel electrophoresis was performed with a Phast gel gradient 10-15 and Phast gel SDS buffer strips

employing the Phast System (Pharmacia LKB Biotechnology).

*Analysis of SDS-PAGE using NIH Image program*

The bands on SDS-PAGE were scanned into a Power Macintosh computer and their images were analyzed using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health).

*Western blotting*

Transfer of protein from the gels onto paper was performed according to the method of Towbin et al.[11] using the Phast system. The protein on the polyacrylamide slab gel was transferred to a nitrocellulose membrane (0.45$\mu$m pore size in roll form, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) according to the principle of electrotransfer experiments. Following completion of the transfer, the blotting paper was washed three times with Dulbecco's phosphate-buffered saline containing 0.05% Tween 20 (pH 7.2) (PBST) at each step. After the initial washing, the blotting paper was soaked in Dulbecco's PBS containing 1% skimmed milk for 1 h at 37℃. The blotting paper was washed again and then soaked in POD-conjugated goat anti-rat myeloma IgE (Fc), diluted 1:1000 with Dulbecco's PBS containing 1% skimmed milk. The blotting paper was incubated for 1 h at room temperature, washed, and then soaked in a solution of 250$\mu$g/ml of 3,3'-diaminobenzidine tetrahydrochloride-0.01% $H_2O_2$-50 mM Tris-HCl (pH7.2). The reaction was stopped after 15 min by washing with distilled water. The blotting paper was finally dried with filter paper.

## RESULTS

*Purification of IgE from serum of rats sensitized with DNP-As*

At each purification step, the IgE contained in the materials was detected by the PCA reaction, and the absorbance at 280nm of the materials showing positive PCA reaction are illustrated in Table 1. The procedure for the purification of rat IgE was selected on the basis of observations of other classes of immunoglobulin contaminating the materials (Table 1). Serum of the Wistar rats sensitized with DNP-As was precipitated with ammonium sulfate at 50% saturation at 4℃, redissolved in PBS, and extensively dialyzed against PBS. The materials containing rat IgE were further purified by ion-exchange chromatography on DEAE-Sephacel. The adsorbed proteins eluted with a linear gradient up to 0.08 M NaCl (tubes no. 160~235) exhibited PCA activities (Fig.1). The proteins from the fractions on DEAE-Sephacel which had PCA activities were further purified by affinity chromatography on goat anti-rat IgG (Fc)-Sepharose 4B (Fig. 2). Non-adsorbed proteins which exhibited PCA activities were further purified by affinity chromatography on Protein A-Sepharose CL-4B (Fig.3). Subsequently, non-adsorbed proteins which demonstrated PCA activities were applied to a column of Sephadex G-150 (Fig.4). PCA reactions were carried out on the obtained fractions (no. 35~50), and it was found that the protein from fractions no. 38~40 displayed PCA activity (Table 2).

*Immunochemical characterization*

The results of SDS-PAGE and profiles obtained from analysis of SDS-PAGE using NIH Image program are illustrated in Fig.5. In the case of the protein from tubes no. 38~40, a single band was observed at m.w. 130,000. Conversely, in the case of rat myeloma IgE (IR 162) digested with papain, multiple bands were observed at m.w. 200,000, 160,000, and 132,000. On Western blotting, no reaction of the protein from tubes no. 38~40 with POD-

Table 1 Classes of immunoglobulin contaminating the material and absorbance at 280 nm of the material showing positive PCA reaction at each purification step

| Purification step | Protein concentration of applied sample (mg/ml) | Ouchterlony test | | | | | | Absorbance at 280 nm showing positive PCA reaction |
|---|---|---|---|---|---|---|---|---|
| | | $IgG_1$ | $IgG_{2a}$ | $IgG_{2b}$ | $IgG_{2c}$ | IgM | IgA | |
| Whole serum | 70 | + | + | + | + | + | + | 1.0 |
| $(NH_4)_2SO_4$ precipitation | 30 | + | + | + | + | + | + | 0.4 |
| DEAE-Sephacel | 3 | + | + | + | + | − | − | 0.3 |
| Goat anti-rat IgG (Fc)-Sepharose 4B | 3 | − | − | + | + | − | − | 0.2 |
| Protein A-Sepharose CL-4B | 3 | − | − | + | − | − | − | 0.3 |
| Sephadex G-150 | 3 | − | − | − | − | − | − | 0.5 |

+: A precipitin band was observed. −: No precipitin band was observed. Sheep antisera to rat immunoglobulins ($IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_{2c}$, IgM and IgA) were employed in the Ouchterlony test.

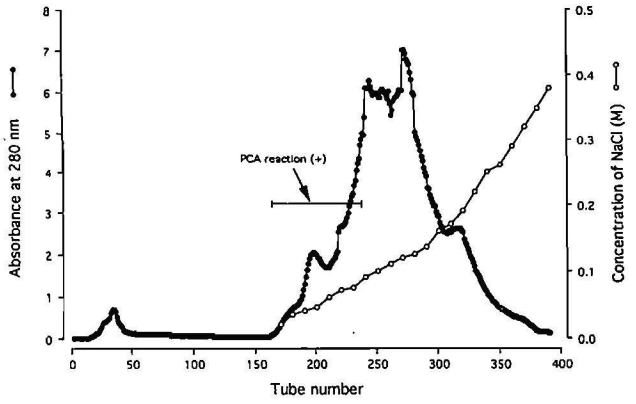


Fig.1 Ion exchange chromatography on DEAE-Sephacel. Column size: 5.0 × 60 cm. Bed volume: 500 ml. Flow rate: 100 ml/h. Fraction size: 20 ml/tube. Starting buffer: 0.05 M Tris-HCl, pH 8.1. Final buffer: 0.05 M Tris-HCl, pH 8.1, 0.8 M NaCl.

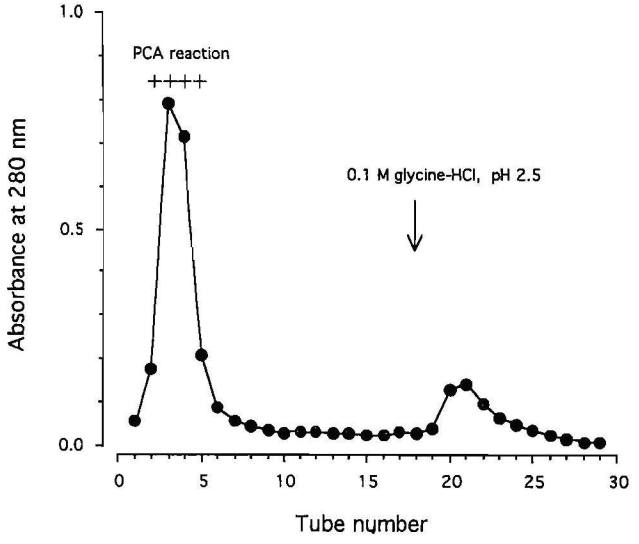


Fig.2 Affinity chromatography on goat anti-rat IgG (Fc)-Sepharose 4 B. Five milligrams of goat anti-rat IgG (Fc) was coupled to 1.5 g of CNBr-activated Sepharose 4B. Bed volume: 5 ml. Flow rate: 20 ml/h. Fraction size: 3 ml/tube. Running buffer: 10 mM PBS, pH 7.4. Eluent: 0.1 Mglycine-HCl, pH 2.5.

goat anti-rat myeloma IgE (Fc) was detected. However, in the case of rat myeloma IgE (IR 162) digested with papain, multiple bands were noted again at m.w. 200,000, 160,000, and 132,000 on Western blotting in which POD-goat anti-rat myeloma IgE (Fc) was employed (Fig.6).

## DISCUSSION

In this study, the protein from tubes no. 38~40 fractionated by gel filtration on Sephadex G-150 displayed PCA activity. The molecular weight of the protein with PCA activity was 130,000 by SDS-PAGE analysis. The protein did not react with POD-goat anti-rat IgG on Western blotting (data not shown), and IgG fractions eluted from anti-rat IgG (Fc)-Sepharose 4B and Protein A-Sepharose 4B did not display PCA activity. It was therefore inferred that the protein obtained in the present study was a

Table 2 Sizes of blue spots in PCA reactions of proteins fractionated by gel filtration on Sephadex G-150

| Sample | Tube no. 38 | Tube no. 39 | Tube no. 40 |
|---|---|---|---|
| Sizes of blue spots (n=2) | 5 × 6 mm<br>5 × 5 mm | 5 × 6 mm<br>5 × 5 mm | 4 × 6 mm<br>5 × 5 mm |

PCA reactions were carried out on fractions no. 35~50. Fractions other than the three fractions indicated did not yield blue spots on rat skin. Concentration of test sample: $A_{280nm}=0.5$.

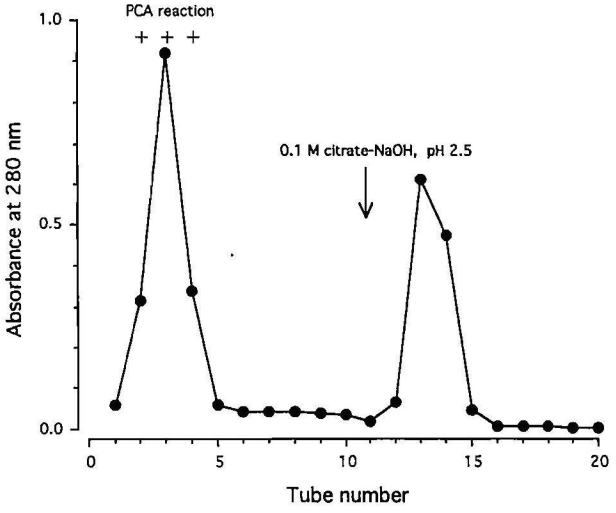


Fig.3 Affinity chromatography on Protein A-Sepharose CL-4B. Bed volume: 7 ml. Flow rate: 20 ml/h. Fraction size: 4 ml/tube. Running buffer: 15 mM PB, pH 8.0. Eluent: 0.1 M citrate-NaOH, pH 2.5.

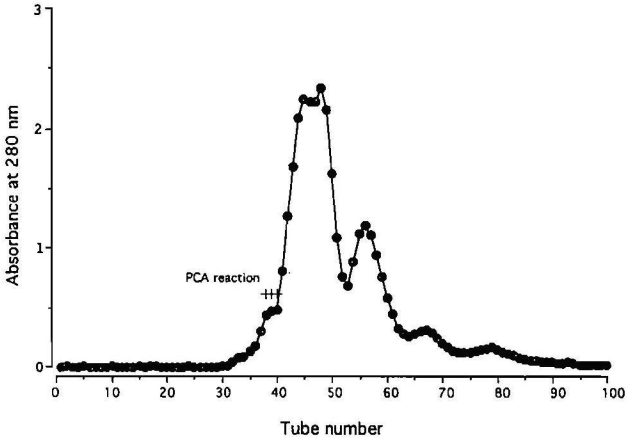


Fig.4 Gel filtration on Sephadex G-150 at the final step of purification of IgE from serum of rats sensitized with DNP-As. Column size: 1.6 × 100 cm. Bed volume: 190 ml. Flow rate: 10 ml/h. Fraction size: 2 ml/tube. Running buffer: 10 mM PBS, pH 7.2.

rat IgE of molecular weight 130,000, but not a rat IgG, and this rat IgE was designated as low molecular weight rat IgE (LMW rat IgE). A low molecular weight (LMW) rat myeloma IgE (m.w. 132,000) was also produced by the digestion of rat myeloma IgE (IR 162) with papain. The molecular weight of the LMW rat IgE almost corresponded to that of the LMW rat myeloma IgE. Rousseaux-Prévost et al. have reported that an $F(ab')_2$-ε (m.w. 130,000) was

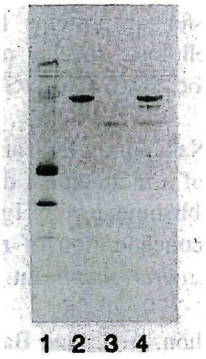


Fig.5-A SDS-polyacrylamide gel electrophoretic analysis of LMW rat IgE. Lane 1, High molecular weight calibration kit proteins (Pharmacia, Uppsala, Sweden) (thyroglobulin, ferritin, catalase, lactate dehydrogenase and albumin); Lane 2, rat myeloma IgE (IR 162); Lane 3, LMW rat IgE; Lane 4, rat myeloma IgE digested with papain (at 37℃ for 10 min). Other details were as described under Materials and methods.

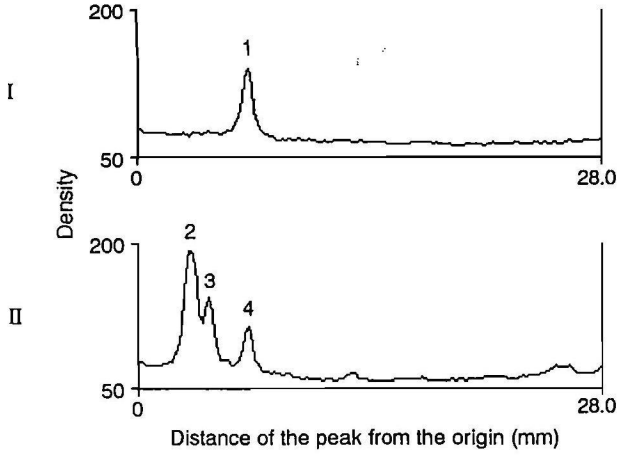


Fig.5-B Profiles obtained from analysis of SDS-PAGE using NIH Image program. I: LMW rat IgE. Peak 1 was observed at molecular weight (mol. wt.) 130,000. II: Rat myeloma IgE digested with papain. Peak 2, 3 and 4 were respectively observed at mol. wt. 200,000, 160,000 and 132,000.

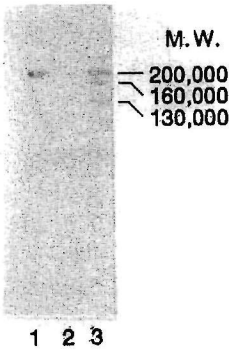


Fig.6 Western blotting analysis of LMW rat IgE. Lane 1, Rat myeloma IgE (IR 162); Lane 2, LMW rat IgE; Lane 3, rat myeloma IgE digested with papain (at 37℃ for 10 min).

obtained by papain digestion of rat myeloma IgE (IR 162)[4]. On this basis, the LMW rat myeloma IgE obtained was considered to be an $F(ab')_2$-ε cleaved from rat myeloma IgE (IR 162). It was therefore inferred that the LMW rat IgE obtained was also an $F(ab')_2$-ε derived from native rat IgE in view of its ability to bind to antigen indicated by PCA reaction. Rousseaux-Prévost et al. found that an $F(ab')_2$-ε (m.w. 130,000) cleaved from rat myeloma IgE (IR 162) was unable to compete for the binding of IgE to rat mast cells *in vitro*[4]. In this study, the results of the PCA reaction on the LMW rat IgE indicated that 18μg of LMW rat IgE yielded a detectable PCA response (extinction coefficient = 1.4[5]). Isersky et al. have reported, however, that 28 ng of rat IgE was sufficient to give a detectable PCA response[5]. Based on these reports and our results, the affinity of the LMW rat IgE obtained for the receptor on mast cells appears to be much lower than that of native IgE. On Western blotting, the LMW rat IgE did not react with POD-goat anti-rat myeloma IgE (Fc). In contrast, the LMW rat myeloma IgE did react with POD-goat anti-rat myeloma IgE (Fc) on Western blotting. Previous reports from our laboratory[12] and others[13~15] have shown that the existence of structural heterogeneity of Fc region within the rat IgE class. From these reports we can speculate that the differences in reactivity of POD-anti-rat myeloma IgE with two LMW IgEs, LMW rat IgE and LMW rat myeloma IgE, on Western blotting are based on the structural heterogeneity of Fc region between two IgEs. However, it remains to be defined which protease degrades IgE proteins in the serum of rat, as the IgE proteins seemed to have been degraded by some proteases in this study. The possibility exists therefore that the above-mentioned results were based on the differences in the proteolytic portion of Fc region between two IgE proteins. It has not yet been determined whether the LMW rat IgE was generated during the process of IgE purification or in the circulating blood *in vivo*. Assuming that the LMW rat IgE obtained in the present study was generated *in vivo*, the possibility exists that the LMW rat IgE may regulate allergic reactions in rats.

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