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An improved method for the detection of IgE FE-3 by ELISA using monoclonal anti-IgE FE-3 antibody

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ABSTRACT

A hybridoma producing monoclonal antibody against monoclonal DNP-specific rat IgE (IgE FE-3) was generated by fusion of P3-X63-Ag8-U1 (P3U1) mouse myeloma and spleen cells from BALB/c mice immunized with IgE FE-3. Five hybridoma clones were obtained. Three clones of them had a high reactivity against IgE FE-3 and a low reactivity against the L-chain of IgE FE-3 on ELISA analysis for the first screening of the hybridoma. The binding of monoclonal antibodies produced from clone BB-9 to IgE FE-3 revealed the strongest inhibition among three monoclonal antibodies on pretreatment with polyclonal rabbit anti-IgE FE-3 antibodies. It was clarified that monoclonal antibody BB-9 reacted specifically with the heavy chain of IgE FE-3 by Western blotting analysis. Furthermore, the reactivity of the monoclonal antibody BB-9 to IgE FE-3 was higher than that of MARE-1, which is a monoclonal antibody against rat myeloma IgE. The monoclonal antibody BB-9 was therefore employed for IgE-capture ELISA to determine the concentration of IgE FE-3. No significant alterations of color development occurred when IgE FE-3 was concomitantly incubated with large excesses of IgG antibodies and rat myeloma IgE. Our data indicated that IgE-capture ELISA employing the monoclonal antibody BB-9 had a higher sensitivity for the determination of IgE FE-3 than did that using polyclonal rabbit anti-IgE FE-3 antibodies. *Ryukyu Med. J., 20(3)129~135, 2001*

Key words: monoclonal antibody against rat IgE, rat IgE, DNP-specific rat IgE, IgE-capture ELISA

INTRODUCTION

We have previously succeeded in determining the concentration of DNP-specific rat IgE in the serum of rats sensitized with DNP-As by means of IgE-capture ELISA employing rabbit polyclonal antibodies against IgE FE-3[1]. However, IgE FE-3 concentrations of less than 10 ng/ml cannot be estimated in vitro by the above method. Heterogeneous antibodies with different antigenic specificities and affinities to the epitope exist in immunized sera, and the composition of the heterogeneous antibodies differs in experimental animals employed for immunization, even if they belong to the same strain[2]. To improve the sensitivity of the previous method, it was considered that a monoclonal antibody against IgE FE-3 needed to be employed for the IgE-capture ELISA to determine IgE FE-3. In the present study, we attempted to produce a monoclonal antibody to IgE FE-3 and to establish a modified method of IgE-capture ELISA for estimating IgE FE-3 using a monoclonal antibody against IgE FE-3.

MATERIALS AND METHODS

Animals

BALB/c mice weighing from 20 to 30 g were employed for the experiments. The mice were purchased from Kyudo Co., Ltd. (Kumamoto, Japan).

Animal care and management

We followed 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 animals, together with the guide for animal experiments issued by the University of the Ryukyus. All animal studies were reviewed and approved by the Animal Care Committee at the University of the Ryukyus.

Reagents

Freund's complete adjuvant was obtained from DIFCO Laboratories (Detroit, Mi. U.S.A.). RPMI 1640 medium was obtained from NISSUI Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Sanko Junyaku (Fukuoka, Japan). 8-Azaguanine was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Polyethylene glycol 4000 (for gas chromatography) was obtained from E. Merck AG (Darmstadt, F.R.G.). The mouse myeloma cell line P3-X63-Ag8-U1 (P3U1) was obtained from American Type Culture Collection (ATCC, Rockville, Maryland., U.S.A.). IgE FE-3, monoclonal DNP-specific rat IgG_{2a}, polyclonal rabbit anti-IgE FE-3 antibodies and L-chain of IgE FE-3 were purified by the same method as described previously[1]. Polyclonal rat IgG was purified by the same method as described previously[3]. DNP-As was purified employing the same method described previously by Strejan *et al.*[4] and Eisen *et al.*[5] Biotinylated DNP-As was prepared according to the method of Nerurkar *et al.*[6] 2,4-Dinitrobenzene sulfonic acid sodium salt was purchased from Tokyo Kasei Inc., Ltd. (Tokyo, Japan). DNP-glycine and 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulfonic acid] (ABTS) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Diethyl ether, Tween 20, 3,3'-diaminobenzidine tetrahydrochloride, Tris (hydroxymethyl) -aminomethane, and gelatin fine powder were obtained from Nacalai Tesque (Kyoto, Japan). Peroxidase (POD)-conjugated goat anti-rat IgG (H+L) and Mouse Typer sub-isotyping panels were obtained from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). Aminohexanoyl-biotin-N-hydroxysuccinimide, POD-conjugated streptavidin, POD-conjugated goat anti-mouse IgG (H+L), POD-conjugated goat anti-rabbit IgG (H+L), monoclonal antibody against rat myeloma IgE (MARE-1), and rat myeloma IgE (IR 162) were obtained from Zymed Laboratories (San Francisco, Calif., U.S.A.). CNBr-activated Sepharose 4B, Protein A-Sepharose CL-4B, Phast gel (gradient 10-15), Sephadex G-200, and Phast gel sodium dodecyl sulfate (SDS) buffer strips were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Unless otherwise stated, only reagent-grade chemicals were used as other reagents.

Cell line

Incubation of P3U1 mouse myeloma cells was carried out according to the method of Nakamura *et al.*[7].

Immunization

Immunization of the BALB/c mice was carried out according to the modified method of Nakamura *et al.*[7]. After the first injection of 100 μg of IgE FE-3 conjugated with Freund's complete adjuvant, into the peritoneal cavity the same amount of immunogen was administered subcutaneously into the femur of each mouse.

Cell fusion

The spleen cells of BALB/c mouse were fused with P3U1 mouse myeloma cells according to the method of Nakamura *at al.*[7]. The culture medium from the hybridoma was collected, and the reactivity of antibody against IgE FE-3 was detected by ELISA.

ELISA for screening of hybridoma producing monoclonal antibody to IgE FE-3

ELISA was performed according to the method of Engvall and Perlmann[8]. Disposable sterile ELISA plates with 96 polystyrene wells (Corning, N.Y., U.S.A.) were coated with IgE FE-3 (1 μg/ml) in Dulbecco's phosphate-buffered saline (PBS, pH 7.4) overnight at 4℃. At this and all other steps except for the blocking step, the wells were filled to a volume of 50 μl. The wells of the plate were washed twice with PBS and incubated for 1 h at room temperature with 200 μl of Dulbecco's PBS containing 1 % skimmed milk as the blocking step. After washing, the culture supernatants from the 384 wells which exhibited growth of cell colonies were added to duplicate wells, and the plate was then incubated for 1 h at room temperature. Following washing with Dulbecco's phosphate-buffered saline containing 0.05% Tween 20 (pH 7.4) (PBST), the duplicate wells were, respectively, incubated with POD-goat anti-mouse IgG (H+L), diluted 1:3,000 with PBST, for 30 min at room temperature. Finally, the wells were washed with Dulbecco's PBS alone. Thirty minutes after the substrate solution (ABTS) had been added to each well, spectrophotometric readings were made using the λ_1:415 nm and λ_2:492 nm wavelength filters of a dual wavelength microplate photometer (MTP-22, Corona Electric Co, Ibaragi, Japan).

Determination of the immunoglobulin class and subclass of mouse monoclonal antibodies

The immunoglobulin class and subclass of the mouse monoclonal antibodies against IgE FE-3 were determined using a Mouse Typer sub-isotyping panel (Richmond, U.S.A.), according to the instruction.

ELISA for determining the specificity of monoclonal antibody against IgE FE-3 produced from the hybridoma

The wells of the ELISA plate were coated with non-reduced IgE FE-3 (1 μg/ml) in Dulbecco's PBS (pH 7.4) overnight at 4℃. After blocking with 1% skimmed milk, the supernatant of the hybridoma with the highest reactivity of antibody against IgE FE-3 was added to the wells. The plate was then incubated for 1 h at room temperature. Following washing with PBST, the wells were incubated with POD-goat anti-mouse IgG (H+L), diluted 1:3,000 with PBST, for 1 h at room temperature. The wells of another ELISA plate were coated with 1 μg /ml of L-chain of IgE FE-3 in Dulbecco's PBS (pH 7.4) overnight at 4℃. Other details of the ELISA procedure were as described under the section on ELISA for screening of hybridoma producing monoclonal antibody to IgE FE-3.

Purification of monoclonal antibodies against IgE FE-3

Purification of the monoclonal antibodies was performed using Protein A-Sepharose CL-4B according to the method of Ey *et al.*[9].

Effects of polyclonal rabbit anti-IgE FE-3 antibody on the binding of monoclonal antibodies against IgE FE-3 to IgE FE-3

The wells of an ELISA plate were coated with 2 μg/ml of DNP-As in Dulbecco's PBS (pH 7.4) overnight at 4℃. After blocking with 1% skimmed milk, 1 μg/ml of IgE FE-3 was added to the wells and incubated for 1 h at room temperature. Following washing with PBST, polyclonal rabbit antibodies against IgE FE-3 serially diluted with PBST were added to the wells, and the plate was then incubated for 1 h at room temperature. Following washing with PBST, the wells were incubated with 1 μg/ml of monoclonal antibodies against IgE FE-3, BB-9, BG-7, and AC-2 diluted with PBST for 1 h at room temperature. Following washing with PBST, the wells were incubated with POD-goat anti-mouse IgG (H+L), diluted 1:3,000 with PBST, for 1 h at room temperature. The inhibition was obtained from the following equation:

Inhibition (%) = [D-(A, B or C)] / D × 100

where A is the absorbance in the case of BB-9, B is the absorbance in the case of BG-7, C is the absorbance in the case of AC-2, and D is the absorbance in the case of PBST without polyclonal rabbit antibodies against IgE FE-3.

Western blotting

Transfer of protein from the polyacrylamide gel onto paper was carried out according to the method of Towbin *et al.*[10] using a Phast system. The protein of non-reduced IgE FE-3 digested with papain and IgE FE-3 treated with 10 mM DTT on the gel was transferred to a nitrocellulose membrane (0.45-μm pore size in roll form; Toyo Roshi Kaisha, Tokyo, Japan) according to the principle of electrotransfer experiments and the modified method of Hanashiro *et al.*[1].

ELISA for assessing the reactivity of monoclonal antibodies against IgE FE-3 to IgE FE-3

The wells of the ELISA plate were coated with 2 μg/ml of DNP-As in Dulbecco's PBS (pH 7.4) overnight at 4℃. After blocking with 1% skimmed milk, 1 μg/ml of IgE FE-3 was added to the wells and incubated for 1 h at room temperature. Following washing with PBST, monoclonal antibody against IgE FE-3 (BB-9) and monoclonal antibody against rat myeloma IgE (MARE-1) were added to the wells, respectively, and the plate was then incubated for 1 h at room temperature. Following washing with PBST, the wells were incubated with POD-goat anti-mouse IgG (H+L), diluted 1:3,000 with PBST, for 1 h at room temperature.

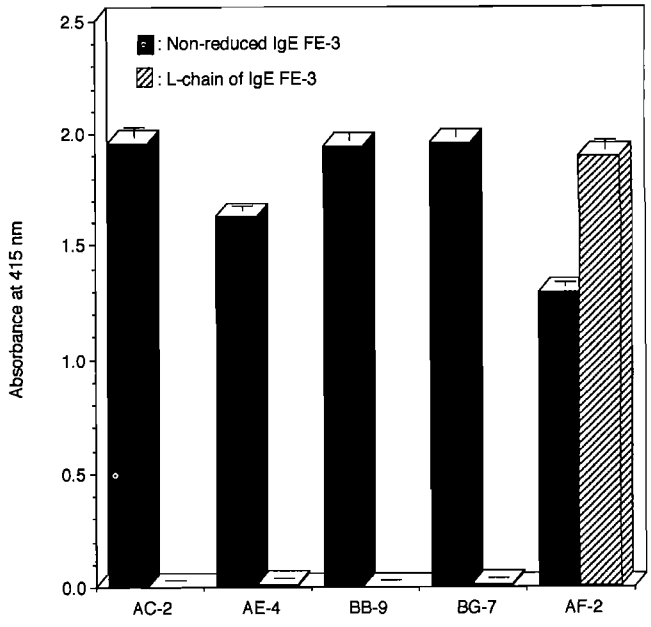


Fig. 1 Generation of hybridoma producing monoclonal antibody against IgE FE-3. Data are expressed as the means ±SD (n=4).

Comparison of the sensitivity on IgE-capture ELISA technique using monoclonal antibodies against IgE FE-3 and polyclonal rabbit anti-IgE FE-3 antibodies

IgE-capture ELISA was performed according to the procedure of Hanashiro *et al*[1]. The wells of the ELISA plate were coated with monoclonal antibodies against IgE FE-3 (5 μg/ml, 2 μg/ml, and 1 μg/ml) and polyclonal rabbit antibodies against IgE FE-3 (1 μg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4℃. After blocking with 1% gelatin-PBS, IgE FE-3 (1 μg/ml) was serially diluted with 1% gelatin-PBST and added to the wells.

Effect of serum protein on IgE-capture ELISA

The ELISA plate was coated with 5 μg/ml of monoclonal antibodies against IgE FE-3 in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4℃. After blocking with 1% gelatin-PBS, the wells were incubated with 1 μg/ml of IgE FE-3 in the presence of 1% gelatin-PBST, non-sensitized rat serum (× 1/100), polyclonal DNP-specific rat IgG (10 μg/ml), or rat myeloma IgE (IR 162, 1 μg/ml) serially diluted with 1% gelatin-PBST, for 1 h at room temperature.

Effect of high concentration of monoclonal DNP-specific rat IgG_{2a} and rat myeloma IgE (IR 162) on IgE-capture ELISA using monoclonal antibodies against IgE FE-3

The ELISA plate was coated with 5 μg/ml of monoclonal antibodies against IgE FE-3 in 0.05 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4℃. After blocking with 1% gelatin-PBS, the wells were incubated with 20 ng/ml of monoclonal antibodies against IgE FE-3 in the

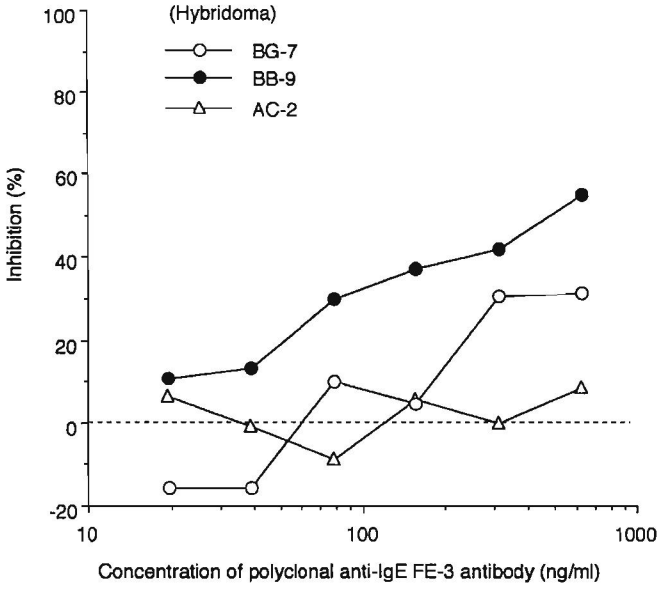
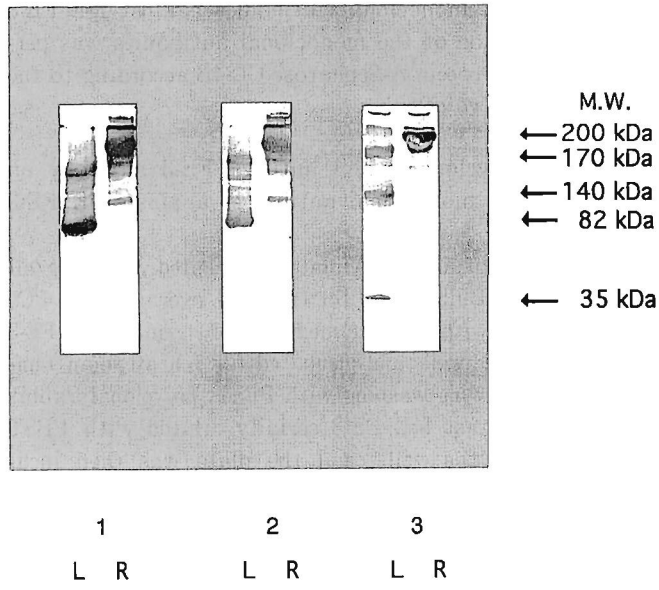


Fig. 2 Effects of polyclonal anti-IgE FE-3 antibodies on the binding of monoclonal antibody against IgE FE-3 to IgE FE-3. ○, BG-7; ●, BB-9; △, AC-2.

presence of monoclonal DNP-specific rat IgG_{2a} (10 ng/ml ~ 10 μg/ml) or rat myeloma IgE (IR 162; 10 ng/ml ~ 100 ng/ml) diluted with 1% gelatin-PBST, for 1 h at room temperature.

RESULTS

After fusion, visible clones were apparent in 321 of 392 wells. Culture supernatans from these wells were analyzed to select hybridoma clones by means of ELISA. Three hybridoma clones, with a high reactivity of IgG antibody against non-reduced IgE FE-3 but a low reactivity of IgG antibody against the L-chain of IgE FE-3, were selected and designated as AC-2, BB-9 and BG-7, respectively (Fig. 1). Three of the culture media from the hybridoma (AC-2, BB-9, and BG-7) were reacted with rabbit anti-IgG_1 antibody and anti-κ-chain antibody. (Data not shown). Furthermore, the experiment was carried out in order to select hybridoma clone with the same binding site of polyclonal rabbit anti-IgE FE-3 against IgE FE-3. The increase in absorbance on ELISA using the monoclonal antibodies, BB-9 and BG-7 was inhibited depending on the concentration of polyclonal rabbit anti-IgE FE-3. The highest values for the inhibitory ratio on ELISA using the monoclonal anti-IgE FE-3 antibodies BB-9 and BG-7 were 55.2% and 30.5%, respectively. On the other hand, the highest value for the inhibitory ratio on ELISA using the monoclonal antibody AC-2 was 9.8% (Fig. 2). From the results of monoclonal anti-IgE FE-3 antibodies BB-9, inhibition with dependence on the concentration of polyclonal rabbit antibodies against IgE FE-3 was observed. Furthermore, Western blotting analysis



Lane

1: MARE-1

(monoclonal antibody against rat myeloma IgE)

2: BB-9

(monoclonal antibody against IgE FE-3)

3: AF-2

(monoclonal antibody against IgE FE)

L: IgE FE-3 treated with DTT

R: Non-reduced IgE FE-3 digested with papain

Fig. 3 Western blotting analysis of purified monoclonal antibody against IgE FE-3, BB-9. SDS-PAGE of IgE FE-3 digested with papain and IgE FE-3 treated with DTT was performed. For the Western blotting, the concentration of protein in each sample was adjusted to 1 μg/ml.

was carried out in order to characterize BB-9. The results of Western blotting analysis for IgE FE-3 treated with DTT are illustrated in Fig. 3. The 200-kDa band of non-reduced IgE FE-3 was present on the electrophoretic patterns of three monoclonal antibodies. In the same patterns for BB-9 and MARE-1, two bands were observed at 170-kDa and 82-kDa in the lane for IgE FE-3 treated with DTT. On the other hand, in the case of AF-2, three bands were observed at 170-kDa, 140-kDa and 35-kDa in the lane for IgE FE-3 treated with DTT (Fig. 3). In order to compare BB-9 and MARE-1 in the reactivity pattern against IgE FE-3, the experiment was carried out using ELISA. The reactivity of the monoclonal antibodies against IgE FE-3, BB-9, to IgE FE-3 was higher than that of MARE-1 (Fig. 4). Furthermore, in order to compare the reactivity of the IgE-capture ELISA technique, the ELISA technique employing BB-9 and polyclonal rabbit anti-IgE FE-3 antibodies, was carried out. The absorbance on the IgE-capture ELISA was correlated with the

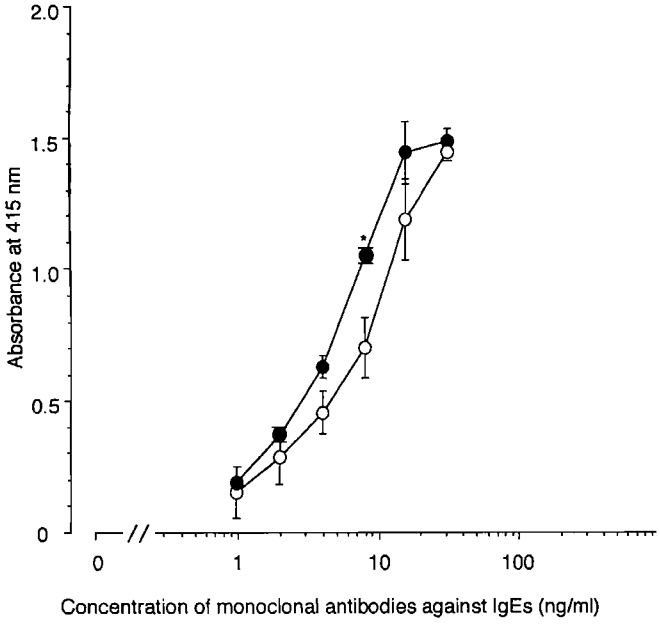


Fig. 4 Assessment of reactivity of monoclonal anti-IgE FE-3 to IgE FE-3. Data are expressed as the means±SD (n=3). *p<0.05 (vs. MARE-1). ○, MARE-1; ●, BB-9.

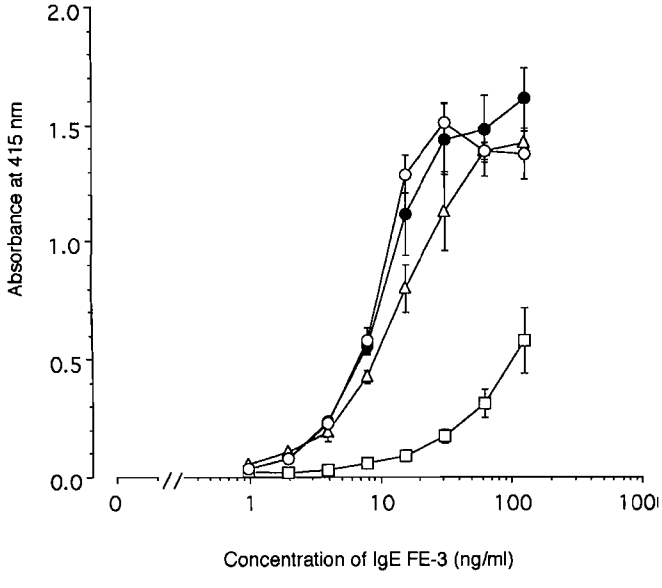


Fig. 5 Comparison of the sensitivity on IgE-capture ELISA technique using monoclonal antibodies against IgE FE-3 and polyclonal rabbit anti-IgE FE-3 antibodies. Data are expressed as the means±SD (n=4). ○, 5 μg/ml of BB-9; ●, 2 μg/ml of BB-9; △, 1 μg/ml of BB-9; □, 1 μg/ml of rabbit polyclonal antibody against IgE FE-3.

concentration of IgE FE-3. The sensitivity and reactivity on IgE-capture ELISA using the monoclonal antibodies against IgE FE-3, BB-9, were higher than those of rabbit polyclonal anti-IgE FE-3 antibody. The highest reactivity of IgE-capture ELISA was seen at a concentration of 5 μg/ml of the monoclonal antibody against IgE FE-3, BB-9

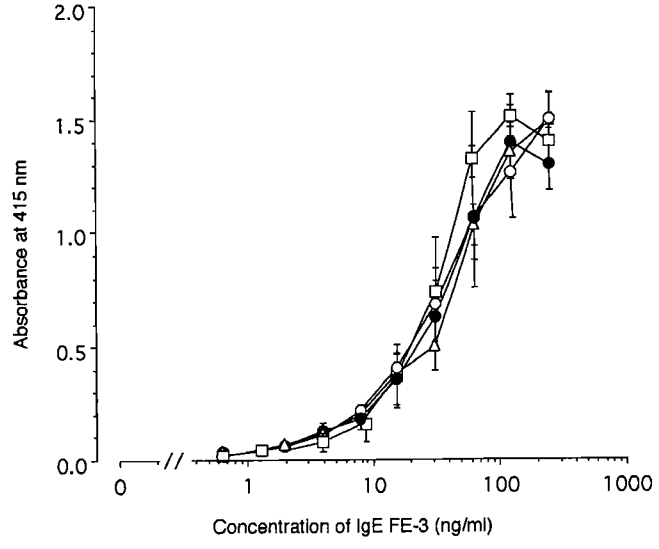


Fig. 6 Effect of serum protein on IgE-capture ELISA. Data are expressed as the means±SD (n=4). ○, 1% gelatin-PBST; ●, non-sensitized rat serum (×1/100); △, polyclonal DNP-specific rat IgG (10 μg/ml); □, rat myeloma IgE (1 μg /ml).

(Fig. 5). The lower detection limit for IgE FE-3 was 2 n g/ml. IgE-capture ELISA using monoclonal antibodies against IgE FE-3, BB-9, was found to be, approximately 10 times higher in reactivity, than that using rabbit polyclonal antibodies. Moreover, the experiments were carried out in order to assess the specificity determing IgE FE-3 on the IgE-capture ELISA technique employing BB-9. The increase in absorbance on IgE-capture ELISA was not interfered by non-sensitized rat serum (× 1/100), polyclonal DNP-specific rat IgG (10 μg/ml), rat myeloma IgE (IR 162, 1 μg/ml) (Fig. 6), or monoclonal DNP-specific rat IgG_{2a} in the concentration range of 10 ng/ml to 10 μg/ml (Table 1). The increase in absorbance on IgE-capture ELISA was inhibited by 1 μg/ml of rat myeloma IgE (IR 162). However, the increase in absorbance on IgE-capture ELISA was not inhibited by rat myeloma IgE (IR 162) in the concentration range of 10 ng/ml to 80 ng/ml (Table 2). These results showed that a monoclonal antibody against IgE FE-3 (BB-9) had a high specificity to IgE FE-3.

DISCUSSION

We have succeeded in developing an IgE-capture ELISA technique by employing monoclonal antibodies against IgE FE-3 (BB-9) in order to determine lower levels of IgE FE-3 as compared to IgE-capture ELISA technique using polyclonal rabbit anti-IgE FE-3 antibodies. We demonstrated that the IgE-capture ELISA technique developed in the present study is highly specific for the determination of IgE FE-3. The data presented in Tables 1 and 2 indicate that no significant alterations of color development occur when IgE samples are incubated with large

Table 1 Effect of high concentration of monoclonal DNP-specific rat IgG_{2a} on IgE-capture ELISA using the monoclonal antibody against IgE FE-3, BB-9, for determination of IgE FE-3

Monoclonal DNP-specific rat IgG_{2a} (ng/ml)	Apparent IgE level (ng/ml)	Inhibition (%)
0	19.9 ±1.76	
10	20.6 ± 1.81	- 3.5 ± 3.02
100	21.0 ± 2.07	- 5.7 ± 2.19
1000	20.0 ±1.68	- 0.5 ± 2.83

Apparent IgE level in the table 1 indicates the concentration of IgE measured by the improved method of ELISA after the addition of IgE FE-3 (20 ng/ml) to the solution contained in monoclonal DNP-specific rat IgG_{2a} at various concentration. The inhibition was obtained from the following equation: Inhibition (%) = (A-B) /A × 100 where A is the absorbance in the case of 20 ng/ml of IgE FE-3 in the presence of PBST, B is the absorbance in the case of 20 ng/ml of IgE FE-3 in the presence of monoclonal DNP-specific rat IgG_{2a} at various concentration. Data are expressed as the means±SD (n=4).

Table 2 Effect of high concentration of rat myeloma IgE (IR 162) on IgE-capture ELISA using the monoclonal antibody against IgE FE-3, BB-9, for determination of IgE FE-3

Myeloma IgE (ng/ml)	Apparent IgE level (ng/ml)	Inhibition (%)
0	20.4 ± 0.94	
10	21.2 ± 0.96	0.4 ± 3.85
20	20.3 ± 0.92	-1.6 ± 4.16
40	19.8 ± 0.92	0.6 ± 3.02
80	19.4 ± 0.47	2.7 ± 2.19

See footnote of table 1. Data are expressed as the means±SD (n=4).

excesses of IgG antibodies and rat myeloma IgE.

It is widely known that MARE-1 is a monoclonal antibody against rat myeloma IgE. MARE-1 is widely used in allergy experiments in rats[11, 12]. Imaoka *et al.* established an IgE-capture ELISA technique for rat IgE antibody to examine the differences in antibody responses against the major allergen of Japanese cedar pollen (Cry j I) between rat strains[12]. However, the reactivity of MARE-1 for IgE FE-3 has been shown to be lower than that of rat myeloma IgE (IR 162)[1]. We therefore attempted to produce a monoclonal antibody against IgE FE-3 which has a high specificity and reactivity to IgE FE-3, in order to establish an IgE-capture ELISA technique with a higher sensitivity for the determination of IgE FE-3 as compared to that using polyclonal rabbit anti-rat IgE antibodies. It was demonstrated that the reactivity of the monoclonal

antibody against IgE FE-3, BB-9, to IgE FE-3 was higher than that of MARE-1 to IgE FE-3. These results and other reports[1, 13, 14] suggest that structural heterogeneity is present in the Fc region of rat IgEs. The possibility exists therefore that a monoclonal antibody against IgE FE-3 with a high reactivity to a particular IgE may have a low reactivity to other IgEs derived from a different origin. On the other hand, IgE is heavily glycosylated and is known to contain N-linked oligosaccharides[15]. Furthermore, there is a different reactivity for differential glycosylation of IgE, since ND myeloma contains 30% more carbohydrate than does PS myeloma[16]. Our data and the above-mentioned reports imply that the heterogeneity in the Fc region of IgE may be dependent on the differential glycosylation of IgE.

On the other hand, the binding site of BB-9 on the IgE FE-3 molecule remains to be clarified. It has been speculated that the binding site of BB-9 is situated in the Fcε3 or Fcε4 region of IgE FE-3, since 1) binding of BB-9 on IgE FE-3 was inhibited by pretreatment with polyclonal rabbit anti-IgE FE-3 antibodies (Fig. 2), and 2) the monoclonal antibody against the C-terminal region of the IgE molecule is suitable for use in IgE-capture ELISA on the basis of the principle of IgE-capture ELISA. Pathophysiological studies in vivo and in vitro on allergic reactions in rats can be precisely performed using the IgE-capture ELISA developed in the present study.

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