Identification of Functionally Different Rat IgE in RBL-2H3 Exocytosis

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ABSTRACT

Background: IgE is closely related to the development of allergies. However, the poor relationship between the specific IgE level and the severity of allergic diseases suggests the possibility of functionally different IgE isoforms. With this in mind, rat basophilic leukemia (RBL)-2H3 activation was analyzed with each type of rat IgE for two parameters, exocytosis and IL-4 mRNA production. RBL-2H3 has been well documented in the rat mucosal mast cell line. **Methods:** RBL-2H3 cells sensitized with each kind of rat IgE was activated by cross-linking FcRI with B5 (monoclonal anti-rat IgE mouse IgG antibodies). The RBL-2H3 exocytosis was measured by analyzing the β-hexosaminidase level, and the level of IL-4 mRNA synthesis was analyzed using semi-quantitative RT-PCR. Rat IgE, which was produced by a parasite infection (REP), was prepared using either *Paragonimus westermani* metacercariae (REP-PW) or *Anisakis simplex* third stage larvae (REP-AS). A rat IgE prototype of IR162 was prepared by a peritoneal injection of immunocytoma. **Results:** The level of exocytosis showed a linear relationship with the rat IgE concentration when REP-PW or REP-AS was applied. However, it exhibited a biphasic response with IR162. In addition, the time course of heating at 56°C illustrated the similarity between REP-PW and REP-AS, which differed from that of IR162. In contrast, the level of IL-4 mRNA synthesis in the RBL-2H3 cells with IR162 was comparable to that of either REP-PW or REP-AS. **Conclusion:** These results suggest that functionally different rat IgE isoforms exists in RBL-2H3 exocytosis. **(Immune Network 2002;2(4):195-201)**

Key Words: Rat, IgE, mast cells, parasites, RT-PCR

Introduction

Several cells such as mast cells/basophils, eosinophils and platelets, which express FcεRI and/or FcεRII on the cell surface, are involved in either the IgE mediated immune responses, or the IgE network (1). Among these, mast cells play a pivotal role in the immune defense (2).

Mast cell activation induced by the cross-linking of FcεRI with the IgE-antigen complex performs rapid exocytosis of cellular metabolites such as histamine, serotonin and a lipid mediator, prostaglandin, which are the factors responsible for triggering the allergic symptoms (3). In addition, the activation accompanies the synthesis of cytokines such as IL-3 IL-4 IL-6 and TNF-α, and the control of the immune reactions (2,4). Although mast cells are activated both by allergic diseases and parasite infections, with the exception of a few kinds of parasites, most infections do not involve allergic symptoms such as urticaria, itching sensations, or a runny nose. The fact that most parasitic infections are less related to allergic diseases means that the profile of IgE produced by parasite infections in mast cell activation requires further investigation (5).

Parasite infections enhance Th2 type immune reactions and IgE production. IgE, which increases characteristically in parasite infections, contributes to the host immune defense against parasites (6). *Paragonimus westermani* is a lung fluke that is distributed worldwide. Its infection is established by eating crayfish or freshwater crabs carrying metacercariae. Although this parasite induces a violent immune reaction and specific

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IgE production (7), the symptoms related to an allergy are not usually observed. *Anisakis simplex* is the parasite of which the host is a marine mammal and its infection is established by eating raw marine fish or cephalopods carrying third stage larvae (L3). In contrast to *P. westermani*, this unusual parasite induces allergic symptoms such as anaphylaxis or urticaria (8).

Allergic diseases are closely related to IgE. However, the small relationship between the level of specific IgE and the severity of the allergy suggests the presence of IgE subtypes (9). Zhang et al. reported that there were several IgE subtypes by alternative splicing to investigate with the relationship with allergy disease manifestation (10). Chan et al. prepared a recombinant form of IgE and activated mast cells. However, no remarkable difference between the IgE subtypes was found (11).

IR162 was the first monoclonal IgE to accelerate IgE research and is regarded as the prototype of rat IgE (12). In addition, rat basophilic leukemia (RBL)-2H3 cells have characteristics of mucosal mast cells and exhibit the most evident exocytosis among the described cell lines (13). Rat IgE, which was produced by a parasite infection (REP), was readily prepared by an oral infection with *P. westermani* metacercariae (REP-PW), or with L3 (REP-AS) (14,15). In addition, monoclonal anti-rat IgE mouse IgG antibodies, such as B5 (16) and mare-1 (17) allowed this research to be performed easily.

In this investigation, each rat IgE were compared in the activation of RBL-2H3 to find any functionally different IgE by analyzing the exocytosed β-hexosaminidase and the synthesis of rat IL-4 mRNA. The rat IgEs used in this study were REP-AS, REP-PW and IR162.

Materials and Methods

Materials. Sprague-Dawley rats (10-week old female) and Balb/c mice (8-week old female, which were purchased at Samyook Animal center (Osan-shi, Korea), were cared for according to the guidelines of the Korea University College of Medicine. The biotinylated mare-1 (mouse IgG anti rat IgE) was purchased from Zymed (South San Francisco, California). The purified IR162 of the monoclonal rat IgE was purchased from either Serotec (Oxford, U.K.) or Zymed, or were prepared from the ascites using the method described by Bazin *et al.* (18) and lyophilized. The ascites were prepared by an injection of the tumor cells into LOU/M rats. The avidin conjugated alkaline phosphatase, sigma 104 of the alkaline phosphatase substrate and *p*-nitrophenyl N-acetyl-β-D-glucosaminide were purchased from Sigma (St. Louis, MO). RBL-2H3 was purchased from ATCC or obtained from NIH (John Rivera's laboratory). These cells were cultured with MEME-Earles supplemented with 15% fetal bovine serum, L-glutamine, sodium pyruvate, and penicillin/streptomycin according to the instructions by the ATCC. B5 (mouse IgG2a anti-rat IgE) ascites from Balb/c mice were prepared using the method reported by Gillette et al (19). B5 ascites were used as prepared in this research or were purified from ascites by precipitation with caprylic acid (20).

Parasite infection and blood collection. The Sprague-Dawley rats were infected with 20 metacercariae of *P. westermani*, using a feeding syringe. The metacercariae were collected from crayfish. The L3 was collected manually in the body cavity of the mackerel purchased from a local market. The infection of L3 per os was accomplished by placing the larvae on the pharynx of rats anesthetized with ether. The serum pools of 4 rats were prepared by bleeding from the ophthalmic plexus vein using an EDTA coated capillary tube (21).

Rat IgE capture assay. The principle of the mouse IgE capture assay used by Campbell et al (22) was applied by replacing the anti-mouse IgE antibodies with the anti rat-IgE antibodies. Briefly, an ELISA plate (Costar, Cambridge, MA, USA) was coated with purified B5 (4μg/ml) in a 100μl coating buffer by incubating overnight at 4°C. One percent bovine calf serum (BCS)/phosphate buffered saline/0.1% Tween 20 (PBST) was used as either the blocking solution or the working buffer. The plate was blocked with the blocking solution by incubation for 1 hr at 37°C and was washed once with PBST and twice with distilled water. The diluted serum samples were added to each well and incubated for 2 hrs in a 37°C incubator. A washed plate was incubated with biotinylated mare-1 diluted to 1 : 1000 for 2 hrs at 37°C. The plate was again washed using the above schedule and incubated with alkaline phosphatase conjugated Extravidin diluted to 1 : 1000 for 2 hrs at 37°C. A sigma 104 (1 mg/ml) substrate was used. The optical Density (OD) was analyzed using a Spectromax ELISA reader at 405/650 nm. The level of each type rat IgE was determined by a comparison with a standard curve. The standard curve for the rat IgE was prepared using the rat IgE of IR162 purchased and calibrated with a sigmoidal graph using the Softmax program (Molecular Devices, Menlo Park, California).

Activation of RBL-2H3 cells with non-specific rat IgE. The activation of RBL-2H3 was done using the method described by Ortega Soto *et al.* (23). In this study, B5 was added instead of dinitrophenyl conjugated bovine serum albumin. The RBL-2H3 cells were placed in a 96 well cell culture plate (Costar, Cambridge, WA) by 10^5/well the day before activation and incubated

in a humid 37°C CO_2 incubator. A tyrode buffer solution (1.8 mm $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$ 2.7 mM KCl, 136.0 mM NaCl, 0.3 mM NaH_2PO_4, 5.5 mM D-Glucose, 12 mM $NaHCO_3$, pH 7.4) was used in these experiments as a working buffer. The working buffer was added to each well by pouring down the sidewall to decrease the spontaneous release. The plate was washed with tyrode buffer once and incubated with each type of rat IgE 100µl/well for 2 hrs in a humid 37°C 5% CO_2 incubator. The plate was washed with a tyrode buffer twice, and again incubated with the B5 antibodies for 2 hrs in a humid 37°C 5% CO_2 incubator. The concentration of B5 ascites was 50µg/ml through this research, otherwise indicated else.

Measurement of β-hexosaminidase release. The level of β-hexosaminidase release was measured using a substrate of *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as an indicator of RBL-2H3 exocytosis. The protocol reported by Ortega Soto *et al.* was applied with some modifications (23). The substrate was dissolved in a citrate buffer (40 mM, pH 4.5) to a concentration of 10 mM, aliquoted, and stored at 20°C. After 2 hrs of incubation in the presence of B5, 20µl of the supernatant from the activated RBL-2H3 cells was transferred to an ELISA plate carrying 50µl of the substrate/well. The reaction was stopped after 2 hrs incubation in a humid 37°C incubator by adding a glycine solution (0.2 M pH 10.8), 50 ul/well. The optical Density (OD) was analyzed with a Spectromax ELISA reader at 405/650 nm (24).

RT-PCR. The production of rat IL-4 mRNA was measured using semi-quantitative RT-PCR in order to verify the induction of mRNA production in the activated RBL-2H3 cells. The following primers for the rat IL-4, which were used by Siegling et al (25), were applied: for the sense ATGCACCGAGATGTTTGTACC, for the antisense TTTCAGTGTTCTGAGCGTGGA. β-actin was used as a house keeping gene ACTCCTACGTGGGCGACGAG was used as the sense primer, and CAGGTCCAGACGCAGGATGGC was used as the sense primer (26). For these experiments, the RBL-2H3 cells were placed at 6 well plates the day before the experiment. The cells were activated with 600µl of each sample per well. The supernatant was removed and the plates were stored at -70°C until needed. RNA extraction from the mammalian cells was performed using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription of the RNA was done using 3µg RNA, 0.5µg oligo $dT_{(15)}$, 1 U of reverse transcriptase (Superscript First Stand System, Gibco-BRL), and a reverse transcription buffer in a total volume of 25µl. One µl of the reverse transcription product was amplified in a 25µl reaction volume containing 25 mM of dNTP, 0.8 uM of each primer, 2.5 ul 10 fold PCR buffer and 1 U BioTaq polymerase (Bioline, Springfield, USA). For the rat IL-4, the PCR reaction was subjected to 29 cycles of denaturation (94°C 1 min), annealing (62°C 1 min) and extension (72°C 1 min) with an initial denaturation step (94°C 5 min), using the PTC-100™ thermal cycler (MJ Research Inc, Watertown MA). The same PCR reaction schedule was used for rat β-actin except the annealing temperature was 59°C and there were 27 thermal cycles. The amplified PCR products were electrophoresed on 2.8% agarose gel and visualized by ethidium bromide staining. Each sample was quantified by densitometry using the Eagle Eye System (Stratagene, La Jolla, California).

Results

Dose dependent response of RBL-2H3 cell activation. To select the optimal condition of RBL-2H3 cell activation, the B5 dose-relationship was determined at a concentration of 0.5 ug/ml rat IgE, at which more than half of the FcεRI on the surface of the RBL-2H3 cells were occupied (27). When the relationship between the β-hexosaminidase release and the amount of B5 was analyzed, the highest activation was gained with 50µg/ml B5 ascites when the concentration ranging from 1.5 to 200 ug/ml was applied (Fig. 1). For the dose-dependent relationship between the exocytosis and the rat IgE, 50µg/ml of the B5 ascites was added to each well of a 96-well plate where RBL-2H3 cells were reacted with each type of rat IgE, ranging from 0.1 to 8 g/ml for IR162, from 0.031 to 2µg/ml for REP-AS and from 0.031 to 1 µg/ml for REP-PW (Fig. 2). The results showed a dose-dependent relationship with a relatively high linear correlation when REP-AS or REP-PW was used. However, the dose dependent response with IR162 exhibited a form of biphasic response.

Inactivation of rat IgE. Rat IgE is a heat labile protein, and the extent of IR162 binding to FcRI decreased when inactivated by heat treatment at 56°C (16). The inactivation profile of the rat IgE was analyzed in the time course. As shown in Fig. 3, RBL-2H3 activation with REP from both parasites was completely abolished when inactivated for 1 hour. However, activation with the inactivated IR162 was still found after 1 hr of the inactivation procedure.

RT-PCR for rat IL-4 transcript production. IL-4 mRNA production was analyzed using semi-quantitative RT-PCR in order to delineate cytokine synthesis. In order to clarify the level of IL-4 mRNA production, the condition in which a linear relationship between the cDNA template dose and the PCR-product occurred

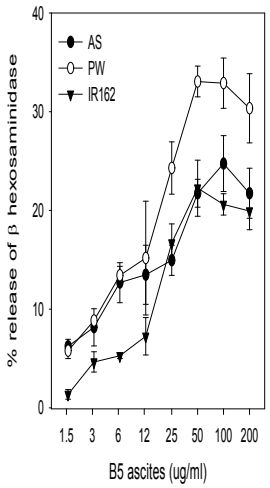

Figure 1. B5 dose response relationship in β-hexosaminidase release. 5×10^4 cells/well in a 96-well plate sensitized with each rat IgE were incubated with diluted B5 for 2 hrs at 37°C 5% CO_2 incubator. 500 ng/ml of at IgE was applied. IR162: monoclonal rat IgE. PW: serum pool from 4 rats infected with the metacercariae of *Paragonimus westermani*. AS: serum pool from 4 rats infected with the third stage larvae of *Anisakis simplex*. Standard Error: SE. SE means the result of triplicate wells of RBL-2H3 cell.

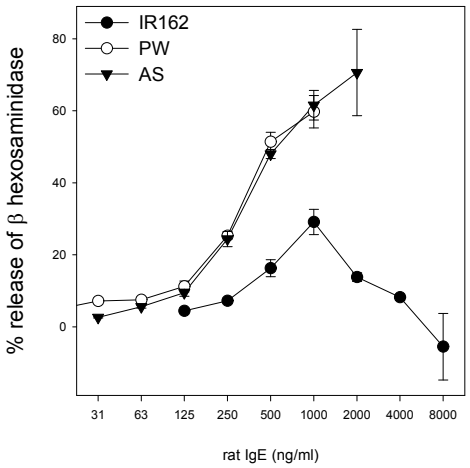

Figure 2. Rat IgE dose response relationship in β-hexosaminidase release. 5×10^4 cells/well in a 96-well plate were incubated for 2 hrs at 37°C 5% CO_2 incubator with B5 ascites diluted to 50μg/ml. IR162: monoclonal rat IgE. PW: serum pool from 4 rats infected with the metacercariae of *Paragonimus westermani*. AS: serum pool from 4 rats infected with the third stage larvae of *Anisakis simplex*. Standard Error: SE. SE means the results of triplicate wells of RBL-2H3 cells.

was selected. The results observed with the 96-well plate was repeated with each rat IgE when the level of exocytosis was analyzed with the cells placed in

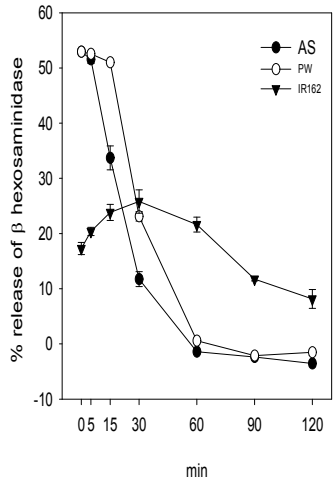

Figure 3. Kinetic analysis of rat IgE inactivation. 10^5 cells/well in a 96-well plate were incubated for 2 hrs in a 37°C CO_2 incubator with B5 ascites diluted 50 ug/ml. IR162: monoclonal rat IgE. PW: serum pool from 4 rats infected with the metacercariae of *Paragonimus westermani*. AS: serum pool from 4 rats infected with the third stage larvae of *Anisakis simplex*. Standard Error: SE. SE means the result of triplicate wells of RBL-2H3 cell.

a 6-well plate. Mast cell activation either with REP-AS or with IR162, as referred in materials and methods, showed the elevation in rat IL-4 mRNA production in comparison with that using tyrode. In contrast to exocytosis, the IL-4 mRNA level from IR162 was comparable to that from REP-AS even when the exocytosis with IR162 was low at high rat IgE concentrations (4 ug/ml). In addition, the level of IL-4 mRNA with REP-PW was comparable to that with REP-AS even though the level of exocytosis was similar (data not shown).

Discussion

Multiple forms of human IgE are generated by alternative splicing at the carboxyl terminus (28). This finding has led to research on the rat IgE profile in order to determine any functional difference in mast cell activation depending on the type of parasitic infection.

RBL-2H3 is a well-documented rat mucosal mast cell line that is derived from a Wistar rat fed β-chlorethylamine (29,30). In this study, the activation of RBL-2H3 cells was established with monoclonal anti rat IgE to obtain reproducible results by binding the monoclonal anti rat IgE to the same site in a rat IgE molecule. In contrast to mare-1 which binds to the Fc fragment of rat IgE and inhibits the binding of rat IgE to FcRI (17), B5 binds to the Fab fraction of rat IgE and does not disturb the binding between

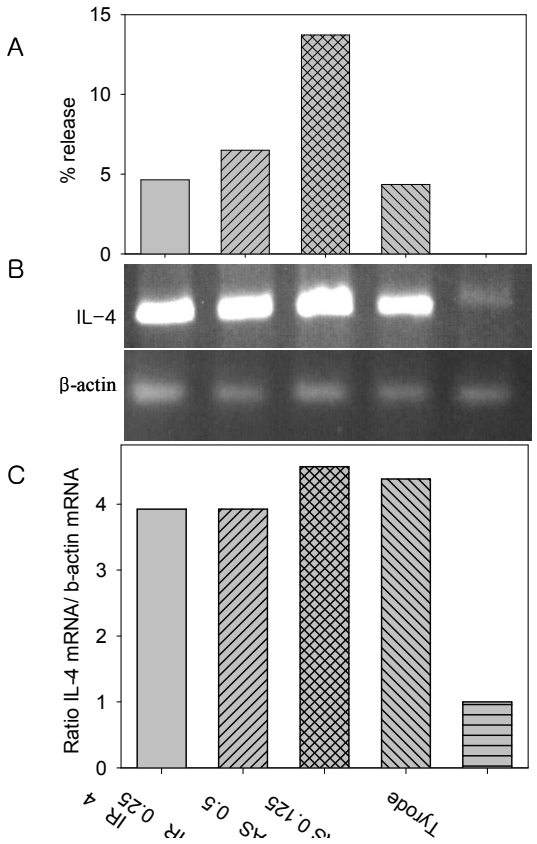

Figure 4. β-hexosaminidase release assay and RT-PCR analysis for rat IL-4 RNA synthesis. Each RNA was extracted from the RBL-2H3 cells activated with rat IgE and B5. Line 1 with IR162 4μg/ml: Line 2 IR162 0.5μg/ml: Line 3 with rat IgE induced by Anisakis simplex third stage larvae (REP-AS), 0.5μg/ml. Line 4 with REP-AS 0.125μg/ml. Line 5 with the Tyrode solution only. A was for the β-hexosaminidase release assay. B was for the RT-PCR products of the IL-4 mRNA and β-actin mRNA stained with ethidium bromide. C showing the ratio of IL-4 mRNA RT-PCR product density over β-actin mRNA RT-PCR product density.

rat IgE and rat FcεRI (16). The letter is believed to cause the cross-linking of FcεRI through the rat IgE and induce the antigen binding to the IgE.

Taurog et al first described a method to activate RBL-2H3 cells with anti rat IgE antibodies and IR162, and has shown a dose-dependent relationship with the cross-linker equivalent to B5, but not with IR162 (31). For the dose relationship with IR162 investigated in this study, there was a rapid decrease at high IR162 concentrations (4 ug/ml) repeatedly in the 3 experiments, as shown in Fig. 2. It is believed that the abrupt decrease at high IR162 concentrations was not the result of any cytotoxic preservatives (32) because even the IR162 dialyzed against phosphate buffered saline exhibited a similar type of decrease. In addition, the IR162 prepared in the laboratory, which did not contain any preservatives, showed a similar rapid slope at 4μg/ml. In contrast, the high linear relationship with REP was reproduced in 5 experiments (Fig. 2).

As the cells interact with T cells bi-directionally, cytokine production in the mast cells affects various aspects of the immune response (4). The results shown in Fig. 4 were reproduced in 3 experiments (data not shown), and it is believed that the cytokine production by RBL-2H3 was available, even when active exocytosis by the FcεRI triggering was not established.

IR162 was the first rodent IgE to be identified, and its isolation was very important in developing the rat and mouse system for analyzing the mechanisms involved in allergic mediator release (12,33). However, the results of this study showed that different IgE preparations can be considerably more efficient with respect to inducing mast cell exocytosis than this prototypic IgE, although IL-4 mRNA production was comparable. Understanding these differences will require structural analyses of the parasite specific IgE molecules. However, the results clearly indicate that the IgE produced *in vivo* will contribute the understanding of the IgE network (1), and that in order for accurate conclusions to be drawn, the efficiency of mast cell activation should be determined with multiple IgE preparations and multiple activation parameters.

As discussed by Yazdanbakhsh et al, a parasite infection is usually chronic and has a life time duration. In this chronic infection, slowly maturing parasites reach the target organs and induce the secretion of immune suppressing cytokines such as TGF-β and IL-10 (34). In a rat infection of *P. westermani*, relatively long and mature worms have been found in 50% of infected rats (35). However, L3 is relatively short and does not mature in the gastrointestinal wall when it infects land mammals (15,36,37). Although no functional differences between REP-AS and REP-PW were found in this research, the underlying mechanism is determined by whether or not the mast cell state is affected by the duration of the parasite infection with those kinds of cytokines. In this context, further application of this mast cell activation method with REP and B5 would be favorable, because the underlying mechanism can be clarified by investigating the relationship between the rat mast cell state and the duration of the parasite infection, and because the activation method showed a high co-linear relationship in contrast to that found with IR162.

In conclusion, these results suggest that there is functionally different rat IgE isoforms in RBL-2H3 exocytosis. In addition, a study on the mast cell activation profile upon parasite infection by applying B5 monoclonal antibodies as the activating tools to

the system is recommended.

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