Receptor destroying enzyme (RDE) from *Vibrio cholerae* modulates IgE activity and reduces the initiation of anaphylaxis

Tatsuya Yamazaki¹, Masanori Inui¹, Keiko Hiemori², Susumu Tomono¹, Makoto Itoh¹, Isao Ichimonji¹, Akina Nakashima¹, Hidekazu Takagi¹, Mrityunjoy Biswas¹, Kumi Izawa³, Jiro Kitaura³, Teruko Imai⁵, Nobuo Sugiura⁶, Hiroaki Tateno², and Sachiko Akashi-Takamura¹*

¹Department of Microbiology and Immunology, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan, ²Biotechnology Research Institute for Drug Discovery National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan, ³Atopy Research Center, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan, ⁴Division of Cellular Therapy/Division of Stem Cell Signaling, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan, ⁵Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-Honmachi, Kumamoto 862-0973, Japan, ⁶Institute for Molecular Science of Medicine, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan.

Running title: RDE modulates IgE to not induce anaphylaxis

*To whom correspondence should be addressed: Prof. Sachiko Akashi-Takamura, Department of Microbiology and Immunology, Aichi Medical University, School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan, Phone: +81-561-62-3311, Fax: +81-561-63-3645, E-mail: sachiko@aichi-med-u.ac.jp

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**Abstract**

IgE plays a key role in allergies by binding to allergens and then sensitizing mast cells through the Fc receptor (FcεRI), resulting in the secretion of proinflammatory mediators. Therefore, IgE is a major target for managing allergies. Previous studies have reported that oligomannose on IgE can be a potential target to inhibit allergic responses. However, enzymes that can modulate IgE activity are not yet known. Here, we found that commercial receptor destroying enzyme (RDE) (II) from *Vibrio cholerae* culture fluid specifically modulates IgE, but not IgG, and prevents the initiation of anaphylaxis. RDE (II)-treated IgE cannot access its binding site on bone marrow-derived mast cells (BMMCs), resulting in reduced release of histamine and cytokines. We
RDE modulates IgE to not induce anaphylaxis

also noted that RDE (II)-treated IgE could not induce passive cutaneous anaphylaxis in mouse ears. Taken together, we concluded that RDE (II) modulates the IgE structure and renders it unable to mediate allergic responses. To reveal the mechanism by which RDE (II) interferes with IgE activity, we performed lectin microarray analysis to unravel the relationship between IgE modulation and glycosylation. We observed that RDE (II) treatment significantly reduced the binding of IgE to *Lycopersicon esculentum* lectin, which recognizes poly-N-acetylglucosamine (GlcNAc) and poly-N-acetyllactosamine (LacNAc). These results suggest that RDE (II) specifically modulates branched glycans on IgE, thereby interfering with its ability to induce allergic responses. Our findings may provide a basis for the development of drugs to inhibit IgE activity in allergies.

Immunoglobulin ε (IgE) plays a key role in type I allergy (e.g. asthma, anaphylaxis). IgE was discovered about 50 years ago by Ishizaka *et al.* as novel immunoglobulin able to induce allergic reactions in the skin (1, 2). The study found that intracutaneous injection with only 1 to 2 ng/ml IgE could induce a erythema-wheal reaction in healthy subjects (2). Mast cells and blood basophils in the tissue were found to express a high affinity to the IgE receptor, FcεRI (3, 4). By subsequent exposure to the allergen, IgE-binding mast cells released proinflammatory mediators, including histamine and cytokines, which cause an allergic response (5). Yamaguchi *et al.* then demonstrated that IgE enhances the expression level of FcεRI in mast cells and permits mast cells to increase production of proinflammatory mediators by antigen challenge (5). Taken together, IgE is considered one of the major targets for therapy against allergy. Omalizumab, which binds to the Fc region of IgE and inhibits binding to FcεRI, has been previously found to be a successful therapy against certain allergies (3, 6, 7). However, omalizumab cannot displace IgE bound to FcεRI, which leads to a delay of several weeks or months before the onset of any clinical benefits (7). As such, another approach to IgE is necessary in order to develop a therapy against allergy.

Glycosylation of immunoglobulin is considered to be important for its structure and function (8). Minor modifications of glycans on IgG (e.g. fucose depletion (9)) can have a significant impact on receptor binding and the effector functions (8). On the other hand, IgE is the most heavily glycosylated antibody (10, 11). Human IgE has seven predicted N-glycosylation sites, whereas murine IgE has eight or nine (8, 10). Björklund *et al.* reported that N-linked glycan is important for binding to FcεRI (12). They indicated that *Flavobacterium meningosepticum* Peptide-N-glycosidase F (PNGase F), which removes almost all N-linked oligosaccharides, reduces the binding level of IgE to FcεRI in ELISA. Moreover, Shade *et al.* reported that oligomannose on N394 in human IgE and N384 in murine IgE are important for the structural integrity of the immunoglobulin (10). Modifications at these sites by endoglycosidase F1 (Endo F1), which cleaves within the chitobiose core of high-mannose and some
RDE modulates IgE to not induce anaphylaxis

hybrid oligosaccharides from N-linked glycoproteins, abrogate IgE binding to FcεRI. Therefore, the modification of oligomannose prevents the initiation of anaphylaxis by mast cells. In their study, they suggested that IgE oligomannose may be a potential therapeutic target. Wu et al. also determined high-mannose glycans on the same site in IgE obtained from a patients with a novel hyper IgE syndrome (11). However, PNGase F and Endo F1 cannot specifically modulate IgE because most sugar proteins have N-linked oligosaccharides. To target the glycan on IgE specifically for the therapy, more research is required to identify a specific enzyme that is able to modulate the glycan structure in IgE.

Previously, we coincidentally found that commercial receptor destroying enzyme (RDE) (II) from *Vibrio cholerae* culture fluid (13) reduced the binding level of IgE to influenza virus antigen, hemagglutinin (HA) (14, Fig. 1A). RDE (II) contains a neuraminidase (NA) (sialidase) and is usually used for preventing non-specific binding of influenza HA to sialic acid in neutralizing assays. We genetically switched the constant region of anti-HA IgG to anti-HA IgA, IgM, IgD, and IgE and established all class of anti-HA-coding plasmids. All variable regions were the same and the binding affinity to influenza HA was considered as equal. However, RDE (II)-treated IgE could not neutralize virus infection despite there being sufficient activity of RDE (II)-treated IgG, IgA, and IgM (14). In the current study, we showed that RDE (II) altered the structure of IgE and abrogated IgE binding to FcεRI on mast cells, which is directly related to the mast cell degranulation, thereby preventing anaphylaxis.

Furthermore, we identified that RDE (II)-treated IgE had reduced capacity of binding to *Lycopersicon esculentum* (LEL), which recognizes poly N-acetylglucosamine (GlcNAc) and poly-N-acetyl-lactosamine (LacNAc) (15–18), by lectin microarray analysis using 96 lectins (19, 20). Taken together, RDE (II) was found to have the specific characteristics necessary to modulate the glycan on IgE, when compared with IgG.

Results

**RDE (II) reduces the binding activity of anti-HA IgE to the antigen and the antibodies against the constant region, but not anti-HA IgG.**

We previously generated the plasmid vector coding antibody-gene of anti-HA IgG and anti-HA IgE (14). Surprisingly, even the variable regions were conserved, wherein anti-HA IgE was not able to neutralize the influenza virus *in vitro* (14). For the neutralizing assay, the specimens were treated with RDE (II) (13), followed by incubation with influenza virus in the presence of trypsin, which cleaves the HA of the influenza virus (21). We also reconfirmed that the antigen-binding activity of anti-HA IgE treated with RDE (II) was reduced to the background level, although anti-HA IgG was not almost affected (Fig. 1A) (14). Moreover, we were almost unable to detect RDE (II)-treated IgE by antibodies against ε chain, despite RDE (II)-treated anti-HA IgG being detected at almost the same level as the untreated antibody (Fig.
RDE modulates IgE to not induce anaphylaxis

1B). In our previous report (14), we could not detect RDE (II)-treated anti-HA IgE using antibodies against ε chain by western blotting. However, the light chain of anti-HA IgG and anti-HA IgE was expressed from the same plasmid vector. Therefore, we measured the binding activity of light chain-binding protein (BP) (Fig. 1C). We detected bands of untreated and RDE (II)-treated IgE at over 250 kDa, which was much larger than predicted, around 200 kDa (3) (Fig. 1C). Taken together, these results suggest that RDE (II) affects not only the antigen-binding region of anti-HA IgE, but also the constant region, except for the light chain. Because anti-HA IgG was not affected by RDE (II), it is also suggested that the reactivity of RDE (II) is specific for anti-HA IgE.

RDE (II) changes the structure of IgE but not IgG, followed by the reduction of the binding activity to anti-ε chain.

To confirm whether RDE (II) affected the IgE antibody, purified mouse monoclonal IgE (anti-TNP IgE) and IgG (anti-T-2 mycotoxin IgG) were treated with RDE (II) and detected by western blotting. Although RDE (II)-treated IgG was detected as several smaller bands, a single band over 150 kDa, which was the intact size of IgG antibodies (Fig. 2A lane 5-8), was detected and identified as the untreated IgG (Fig. 2A lane 1-4). Contrarily, in the absence of RDE (II), the band of the purified IgE was mainly detected as over 250 kDa, as seen in Fig. 1C (Fig. 2A lane 9-12). We also detected a much smaller sized band of RDE (II)-treated IgE, around 150 kDa (Fig. 2A lane 13-16). Moreover, we obtained the same results by CBB staining (Fig. 2B) and light chain BP (Fig. 2C). To analyze which heavy chain (ε chain) or light chain RDE (II) has a great effect, we analyzed RDE (II)-treated IgE under reducing conditions. Using antibodies against the ε chain, we obtained a smaller band (around 50 kDa) than that of untreated (over 75 kDa), depending on the serial dilution (Fig. 2D). Using light chain BP, the band size obtained was the same even after treating with RDE (II) (Fig. 2E). These results indicate that RDE (II) affects the ε chain and not the light chain, which is similar to that demonstrated in Fig. 1C. To confirm the influence of RDE (II) on the constant region, we quantified the binding activity of the antibodies against the constant region in presence and absence of RDE (II). In the plate coated with anti-mouse immunoglobulins (Igs), RDE (II)-treated IgE levels decreased depending on the concentration of RDE (II) (Fig. 2F), although RDE (II)-treated IgG was stably detected at almost the same level as that of untreated (Fig. 2G). Moreover, a 34-fold dilution was the titer of RDE (II) for IgE in the reduction of binding level to coated anti-mouse Igs (Fig. 2F) and that of the band from over 75 kDa to 50 kDa (Fig. 2D and F). Taken together, these results suggest that the reduction of the size by RDE (II) correlates with the binding activity to antibodies against ε chain.

Next, we confirmed whether RDE (II) could reduce the binding activity of serum IgE to anti-ε chain *ex vivo*. We previously succeeded in inducing the expression of anti-HA IgE in the mouse serum by hydrodynamic injection with the plasmid vector encoding the antibody gene
RDE modulates IgE to not induce anaphylaxis

We obtained serum from mice in which anti-HA IgE and anti-HA IgG antibody coding gene had been transferred, followed by incubation with RDE (II). The expression level of RDE (II)-treated anti-HA IgE was not detected in the serum at all, although 2400 ng/ml (=10^{3.4}) of the untreated specimen was detected (Fig. 3A). On the contrary, RDE (II)-treated anti-HA IgG in serum was detected at the same level as that in the absence of RDE (II) (Fig. 3B). We also confirmed that the entire level of IgE in the mouse serum was reduced by treatment with RDE (II) (Fig. 3C). Contrarily, the entire IgG level was the same as the level both of RDE (II)-treated and untreated specimens (Fig. 3D). These results indicate that RDE (II) specifically reduces the binding of IgE to anti-ε antibodies but does not affect the level of IgG in serum. Therefore, this suggests that RDE (II) influences the construction of the ε chain, not the γ chain.

RDE (II)-treated IgE could not induce anaphylaxis in bone marrow-derived mast cells (BMMCs).

RDE (II)-treatment affects the construction of IgE. Therefore, we investigated whether RDE (II) alters IgE/Ag-mediated mast cell activation. To evaluate the stimulation and release of cytokines and histamines from BMMCs with RDE (II)-treated IgE, BMMCs were incubated with RDE (II)-treated anti-dinitrophenol (DNP) IgE (22). Furthermore, BMMCs were activated by cross-link of IgE with DNP-human serum albumin (HSA). The supernatants were obtained and the induction level of TNF-α, IL-6, and histamine were measured, which are the classical activation marker of mast cells. Although untreated IgE-sensitized BMMCs released large amounts of TNF-α, IL-6, and histamine, all of the levels were significantly reduced in RDE (II)-treated IgE-sensitized BMMCs (Fig. 4A-C). To confirm the binding of RDE (II)-treated IgE to BMMCs, we sensitized the cells with untreated or RDE (II)-treated anti-DNP IgE. As shown in Fig. 4D, although untreated IgE could bind to BMMCs, RDE (II)-treated IgE could barely bind to BMMCs (Fig. 4D). However, the binding level of control IgG to bone marrow-derived macrophages (BMMs) was barely affected by RDE (II). We also confirmed that RDE (II) was not toxic for BMMCs, because PI positive cells were barely detected in BMMCs with RDE (II)-treated IgE (Fig. S1). These data indicate that RDE (II)-treated IgE can no longer bind to BMMCs. Therefore, BMMCs are unable to release cytokines and histamine even in presence of the antigen.

To evaluate whether RDE (II)-treated anti-DNP IgE has a similar effect in vivo, we examined IgE-mediated passive cutaneous anaphylaxis (PCA) in mouse models. Increased vascular permeability was observed in mice that were injected with untreated anti-DNP IgE (Fig. 4E and F). On the contrary, vascular permeability was significantly decreased in mice with RDE (II)-treated anti-DNP IgE, as in the control (Fig. 4E and F). These results correlated with those observed in the experimental data of BMMC.
RDE modulates IgE to not induce anaphylaxis

Structure of IgE was affected by trypsin, but IgG not, as with RDE (II).

RDE (II) significantly affects the structure and activity of IgE. Therefore, this suggests that the neutralizing titer of anti-HA IgE against influenza virus was reduced in vitro, compared with that of anti-HA IgG in our previous report (14). To cleave the HA of influenza virus, to enable the virus to infect host cells, we incubated the cells in the presence of trypsin (21). We also confirmed the binding activity of the anti-ε chain to the trypsin-treated IgE. Although trypsin-treated IgG was detected at almost the same level as intact IgG (Fig. S2B), trypsin-treated IgE was significantly reduced by 2-fold (Fig. S2A). To evaluate whether trypsin affected the structure of IgE, we separated trypsin-treated IgE by western blotting under non-reducing conditions. Interestingly, the main band of the IgE was reduced to around 150 kDa, as previously seen in RDE (II)-treated IgE (Fig. S2C lane 10-12). On the contrary, trypsin-treated IgG was detected at the same size as untreated IgG (Fig. S2C lane 4-6). Under reducing conditions, we also obtained a smaller band, around 50 kDa, than that of untreated IgE, depending on the serial dilution (Fig. S2D). These results suggest that trypsin also specifically affects the structure of IgE, as RDE (II).

RDE (II) resists several protease inhibitors to modulate IgE.

Both RDE (II) and trypsin significantly reduced the binding activity of IgE against anti-ε chain antibodies and affected the structure. It was considered that RDE (II) has protease activity like trypsin. To confirm our hypothesis, we used pre-incubated RDE (II) at 56°C or 100°C (56°C -RDE (II) or 100°C -RDE (II)), followed by treatment of IgE and IgG. 56°C -RDE (II)-treated IgE indicated the same binding activity to anti-ε chain as intact RDE (II), which was reduced by 2-fold compared with the control (Fig. 5A). On the other hand, 100°C -RDE (II)-treated IgE was not detected in the reduction of the binding activity to the anti-ε chain, at the same level as the control (Fig. 5A).

We also found that a band of ~150 kDa was not detected in 100°C-RDE (II)-treated IgE, and only a band over 250 kDa was detected (Fig. 5B). These results suggest that the activity of RDE (II) is caused by a function of an enzyme such as protease or glycosidase. To remove the possibility of low molecular weight compound-induced function, we dialyzed RDE (II) to exclude any molecule below 12-16 kDa. We confirmed that the band of IgE treated with dialyzed RDE (II) was also around 150 kDa, the same as that treated with intact RDE (II) (Fig. 5C). These results suggest that RDE (II) works as an enzyme for the modulation of IgE. Then, we analyzed the time course of the reduction by treatment with RDE (II). After incubation for 10 min, ladder bands between >250 kDa to ~150 kDa were detected (Fig. 5D lane 2). The band over 250 kDa reduced and that around 150 kDa increased over time (Fig. 5D lane 3-4). After 360 min, only bands around 150 kDa were detected, with almost no bands over 250 kDa (Fig. 5D lane 6). This result demonstrates that the band size of IgE is gradually reduced by RDE (II),
RDE modulates IgE to not induce anaphylaxis

and not abruptly reduced from over 250 kDa to around 150 kDa.

RDE (II) was obtained from the culture fluid of Vibrio cholera, which contains some protease and sialidase (23). To evaluate the protease activity of RDE (II) for the modulation of IgE, we pre-treated RDE (II) with paraoxon, diisopropyl fluorophosphate (DFP), as the serine-protease inhibitor (24), or bis-p-nitrophenyl phosphate (BNPP) as the carboxylesterase inhibitor (25). Paraoxon and BNPP could not inhibit the IgE modification function of both RDE (II) and trypsin (Fig. S3A). On the other hand, DFP could inhibit the modification function of trypsin (Fig. S3B lane 5 and 6), but not of RDE (II) (Fig. S3B lane 3 and 4). These results suggest that RDE (II) activity is not a result of carboxylesterase and serine-protease activity. Next, we evaluated the inhibitory effect against RDE (II) function with another protease inhibitor, cOmplete, which contains several inhibitors (e.g. α2-macroglobulin, see Experimental procedures). cOmplete was also found to inhibit the modification of IgE by trypsin (Fig. S3D), but not RDE (II) (Fig. S3C), regardless of the concentration. In CBB staining, we could not detect any extra bands of IgE by incubation with cOmplete-treated-RDE (II) (Fig. S3E). These results suggest that the function of RDE (II) is mainly not via protease.

**Characteristic features of glycan alteration on RDE (II)-treated IgE.**

RDE from Vibrio cholerae is a known NA (sialidase) (26). We examined the glycan structures of RDE (II)-treated IgE with lectin microarray using two types of IgE (#1; anti-TNP IgE, #2; anti-DNP IgE). To select a lectin probe, the mean-normalized data for untreated and RDE (II)-treated IgE were analyzed by Student’s t-test. The binding activity of RDE (II)-treated IgE for two lectins, LEL and Phaseolus Vulgaris Leucoagglutinin (PHA-L), were significantly reduced ($P<0.01$) (Fig. 6A, Table S1). The binding of LEL was decreased by 3.5- or 7.1-fold after RDE (II) treatment (Fig. 6B), and that of PHA-L by 5.0- or 9.1-fold (Fig. 6C). This result demonstrates that RDE (II) influences LEL- and PHA-L-binding to the sugar chain of IgE. To confirm these results, we also carried out lectin blotting assays. The signal intensity of LEL (Fig. 6D lane 1 and 2) and PHA-L (Fig. 6F lane 1 and 2) was significantly reduced in RDE (II)-treated IgE against each internal control (Fig. 6E and G lane 1 and 2). Interestingly, the band of IgG was not detected with either LEL or PHA-L (Fig. 6D and F lane 3 and 4), even though the band was detected with anti-IgG antibodies (Fig. 6E and G lane 3 and 4). These results suggest that the glycan structures of IgE and IgG are different, which exercises an effect on RDE (II). On the contrary, RDE (II)-treated IgE remained to be detected with several sialic acids (Fig. 6A and Table S1). Unlike in treatment with RDE (II), we almost failed to detect a reduction in the band of IgE by treatment with NA (sialidase) (Fig. S4 lane 3-9), similar to the situation with IgG (Fig. S4 lane 12-18). These results suggest that sialidase is not primarily responsible for the modification of IgE by RDE (II).
RDE modulates IgE to not induce anaphylaxis

The structure of IgE was also affected by trypsin. Therefore, we also analyzed the binding level of trypsin-treated IgE to LEL and PHA-L via lectin blotting. The signal intensity of trypsin-treated IgE with LEL (Fig. S5 left panel), not PHA-L (Fig. S5 middle panel), was significantly reduced (Fig. S5 bar-graph). These results suggest that at least poly-GlcNAc or poly-N-LacNAc, which was recognized by LEL, are important sugar chains in the structure of IgE.

RDE (II) is a potent enzyme and influences the structure of IgE.

Glycan structures, which bind to LEL or PHA-L, are potentially important for IgE structure. Previously, Björklund et al. reported that N-linked glycan in IgE influences the receptor binding structures (12). We confirmed that anti-HA IgE was not detected in the supernatant of HEK293T cells in the presence of tunicamycin, which inhibits N-glycosylation of glycoprotein (Fig. S6A lane 2 and 3). The expression of anti-HA IgG was detected even in the presence of tunicamycin (Fig. S6A lane 5 and 6). This result suggests that N-glycosylation influences the structure of IgE, compared with IgG. We then analyzed whether PNGase F reduces the band size of IgE. Under non-denaturing conditions, PNGase F slightly reduced the band size of IgE, unlike RDE (II)-treated IgE around 75 kDa (Fig. S6B lane 5 and 6). On the contrary, under denaturing conditions, a reduction in the IgE band to ~75 kDa was detected by treating with PNGase F (Fig. S6C lane 5 and 6). Shade et al. also reported that oligomannose, an N-linked glycan, is indispensable for IgE binding to FcεRI. However, we could not confirm that Endo H reduced the IgE band as RDE (II)-treated IgE under both non-denaturing and denaturing conditions (Fig. S6B and C lane 3 and 4). These results suggest that RDE (II) has a more potent effect against even the intact structure of IgE, compared with PNGase F and Endo H.

Discussion

In the current study, we demonstrated that an enzyme from Vibrio cholerae culture fluid (RDE (II)) can modulate only IgE, and not IgG. The results suggest that LEL-binding sugar chains on IgE are important to retain the structure, allowing for binding to antigen and FcεRI on mast cells. In a previous report, IgA and IgM were also found to not be affected by RDE (II) (14). Moreover, we were unable to detect the modulation of light chain by the RDE (II)-like ε chain using western blotting (Fig. 1C, 2C, and 2E). This suggested that RDE (II) can modulate only the ε chain but not the light chain. Using light chain BP, we detected a band of RDE (II)-treated IgE around 150 kDa, which is the same size as the anti-ε chain (Fig. 2A and C). This result indicates that RDE (II) did not separate IgE into an ε chain and a light chain. These results demonstrate that the enzymatic activity of RDE (II) is able to specifically inhibit the ε chain in the initiation of anaphylaxis.

Previous studies have reported that N-glycosylation of IgE is essential for its binding to FcεRI and its function (10, 12). IgE has four constant domains (Cε1-4) and is heavily...
RDE modulates IgE to not induce anaphylaxis

glycosylated (27). Shade et al. reported that the N-linked oligomannose structure on Cε3 of IgE plays a crucial role to abrogate binding to FceRI (10). Therefore, they concluded that mutations of Cε3 or Endo F1 rendered IgE incapable of eliciting mast cell degranulation. However, we could not find this effect of PNGase F and Endo H by western blotting (Fig. S6B). The longer treatment time of 72 h, as seen in Shade et al., may reduce the band size of IgE. Moreover, although our result in Fig. S6C is consistent with previous reports (10, 12), IgE was only affected by PNGase F under denaturing conditions. On the contrary, RDE (II) was found to affect the structure of IgE after only 10 min incubation under non-denaturing conditions (Fig. 5D). These results suggest that RDE (II) activity has a greater effect on the structure of IgE, when compared with PNGase F and Endo H.

The band size of mouse IgE from HEK293T cells was barely affected by RDE (II) (Fig. 1C), possibly due to the glycosylation in human cells (28, 29), although the binding level of anti-ε chain decreased considerably (Fig. 1B). However, RDE (II) decreased the band size of ε chain from mouse hybridoma (C38-2) to approximately 50 kDa, which was smaller than the predicted size of 73 kDa (Fig. 2D). Moreover, any protease inhibitor could not inhibit the effect of RDE (II) (Fig. S3). Taken together, we considered that IgE was not directly digested by RDE (II) but instead primarily affected the sugar chains on ε chain. The current result also suggests that not only one but several glycans on IgE are involved in modulation, since RDE (II) was found to gradually modulate the structure of IgE (Fig. 5D). Furthermore, our results (Fig. 1C and 2C) also suggested that some effects of RDE (II) are different between mouse and human IgE because of the difference in the glycan structure (10).

Via our lectin microarray using 96 lectins, we identified LEL and PHA-L (P<0.01), which decreased the binding level to RDE (II)-treated IgE (Fig. 6). LEL recognizes N-GlcNAc β-1,4-linked N-GlcNAc oligomers up to 4 carbohydrate units ((GlcNAc)\(_{2,4}\)) or Galβ1-4GlcNAcβ1-3 oligomers ((LacNAc)\(_{n}\)) (15–18). PHA-L binds to tetra-antennary complex oligosaccharides (Gal(β1-4)GlcNAc(β1–6)Man). The current result suggests that RDE (II) modulates branched glycans on IgE, which play a crucial role in retaining the structure and function of IgE. Trypsin was observed to have a similar function to RDE (II) in the modulation of IgE (Fig. S2). Interestingly, trypsin-treated IgE was reduced to the binding level of only LEL, not PHA-L (Fig. S5). This result suggests that LEL-binding sugar chains are more important for the modulation of IgE than PHA-L. On the other hand, both lectins were unable to bind to IgG (Fig. 6D and F). This suggests that the glycoform in the IgE structure is different from that in IgG. Taken together, these results suggest that may be possible for RDE (II) to specifically recognize the IgE-glycan, not IgG.

The binding to anti-ε antibody in ELISA and the change of the band size in western blotting were correlated because the titer of RDE (II) was the same (Fig. 2D and F). Therefore, this suggests that modulation of the structure by RDE (II) is important for the inhibition of IgE
RDE modulates IgE to not induce anaphylaxis

function. RDE (II)-treated IgE was also unable to bind to the antigen and was thereby incapable of neutralizing influenza virus infection (14). These results suggest that the enzyme in RDE (II) drastically changed both the Fc region and antigen-binding site of IgE, not IgG. It is possible that IgE is unable to bind not only to the FcεRI receptor but also to the allergen as a result of RDE (II) treatment. Taken together, this indicates that RDE (II) may be able to inactivate the allergic function of IgE.

It is well known that RDE (II) has both protease and sialidase activity (23). However, unlike trypsin, we could not inhibit the function of RDE (II) via any protease inhibitor (Fig. S3). We also found that the main function of RDE (II) was not sialidase, because RDE (II)-treated IgE was still able to bind several sialic acids in the lectin array (Fig. 6A and Table S1) and the band size IgE was not reduced by sialidase (Fig. S4). Vibrio cholerae has other glycosidase activity (30, 31). One is amidase, which is crucial for cell division and growth (30). Amidases release peptide side chains from the glycan strand, followed by the cleavage of septal peptidoglycan (PG), which is composed of glycan chains with alternating β-1,4-linked N-GlcNAc and N-acetylmuramic acid (Mur-NAc) peptide residues (30). Vibrio cholerae also has at least two chitinases, which synergistically hydrolyze chitin, (GlcNAc)$_n$, into small oligomers (32, 33). It is possible that the amidase or chitinase in RDE (II) can modulate the structure of IgE; however, further studies are required to elucidate the relevance of RDE (II) in inhibiting the function of IgE.

One antibody drug, omalizumab, binds to the Fc fragment of IgE and is able to neutralize IgE by blocking the binding to FcεRI (3, 6, 7). Therefore, the target of IgE is highly effective for therapy against allergy. However, omalizumab has a limitation, that is the delay in the onset of clinical benefits which can take several weeks or months because omalizumab is unable to displace IgE bound to FcεRI under the current treatment conditions (7). In the current study, RDE (II) was found to decrease the binding level of not only the Fc region but also at the antigen binding site (Fig. 1A and B). Therefore, RDE (II) may be able to abrogate the function of even the IgE bound to FcεRI by inhibiting the binding of the allergen. Moreover, we also found that RDE (II) had an effect not only in purified monoclonal IgE but also in IgE in the serum (Fig. 3). This suggests that RDE (II) is effective against polyclonal IgE, which have multiple kinds of glycan. In this study, RDE (II) showed specific characteristics for modulating the glycan on IgE, compared with IgG. Taken together, our results suggest that treatment with RDE (II) could be successful for therapy against allergy; however, further studies are needed to identify the specific enzyme in RDE (II), which modulates IgE.

Experimental procedures

Plasmid construction

In our previous report (14, 34), we generated the plasmids encoding the genes for the heavy chain (IgG and IgE) and the light chain (kappa) of a neutralizing anti-hemagglutinin (HA) antibody (35). All constructions are based on the
RDE modulates IgE to not induce anaphylaxis

pCADEST1 vector (36).

Reagents
Receptor destroying enzyme (RDE) (II) produced from *Vibrio cholerae Ogawa* type 558 (Denka Seiken, Tokyo, Japan) was dissolved in 20 ml of 0.9% NaCl solution and stored at -20°C until use. In some experiments, RDE (II) was dialyzed overnight against 100 volumes of PBS(-) using cellulose tubing (Viskase Companies, Inc., IL, USA) with a molecular weight cut-off (MWCO) of 12-16 kDa. One mg/ml acetylated trypsin (Sigma-Aldrich, St Louis, MO, USA) were dissolved in sterilized dH₂O. As demonstrated in Figs. S2A and B, the reaction with trypsin was performed using MEM medium (Nissui, Tokyo, Japan) containing 0.03% glutamine, 0.01 M HEPES, 0.2% BSA, and 0.075% NaHCO₃ as the neutralizing assay, as mentioned in previous reports (14, 34). Neuraminidase (NA) from *Arthrobacter ureafaciens* (Nacalai Tesque, Tokyo, Japan), Endo H, PNGase F (New ENGLAND BioLabs Inc. Beverly, MA), purified mouse monoclonal anti-T-2 mycotoxin IgG (15H6) (Southern Biotech, Birmingham, AL, USA), purified mouse monoclonal anti-TNP IgE (C38-2) (BD Pharmingen, San Diego, CA, USA), and cOmplete (Roche Diagnostics, Mannheim, Germany), which is composed from several protease inhibitors (aprotinin, bestatin, calpain inhibitor, chymostatin, E-64, leupeptin, α2-macroglobulin, pefabloc SC, pepstatin, PMSF, TLCK-HCl, and trypsin inhibitor), were purchased. Bis-p-nitrophenyl phosphate (BNPP) (SIGMA), which is carboxylesterase (CES) inhibitor (25), paraoxon (SIGMA), and diisopropyl fluorophosphates (DFP) (Wako, Osaka, Japan), which are a serine protease inhibitor, were also purchased.

Antibody expression in vitro
Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal calf serum and penicillin–streptomycin–glutamine. HEK293T cells were co-transfected with the plasmid vectors using FUGENE HD Transfection Reagent (Promega, WI, USA). After one week, the supernatants were obtained and treated with RDE (II) at 37°C overnight (12–20 h), followed by incubation at 56°C for 30 min as mentioned in our previous report (14). HEK293T cells were also transfected and incubated in the presence or absence of tunicamycin (Wako). Forty hours later, the supernatants were obtained.

Mice
Balb/c mice (SLC, Shizuoka, Japan) were maintained under specific pathogen free conditions. Animal experiments were conducted in accordance with the approval of the Animal Research Committee of the Aichi Medical University.

ELISA
The antigen-binding level of anti-HA antibody treated with RDE (II) was determined as previously reported (14, 34, 37). Briefly, a 96-well plate was coated with HA protein purified...
RDE modulates IgE to not induce anaphylaxis

from A/PR8 virus using affinity columns constructed by coupling of CNBr-activated Sepharose 4B beads (GE Healthcare UK Ltd, Buckinghamshire, England) and anti-HA antibodies. The plate was incubated with PBS-containing bovine casein (Merck Millipore, CA, USA) for blocking. The plate was then incubated with serially diluted supernatants or serum. HA-specific antibodies were detected by using HRP-conjugated goat anti-mouse IgG (Southern Biotech) or IgE. Anti-HA IgG purified from a hybridoma (35) or anti-HA IgE obtained from the transfected-HEK293T cells were used as the standard (14, 34, 37). Finally, the expression level was detected using an ELISA POD Substrate TMB kit (Nacalai Tesque). Absorbance was measured by Spectramax M5 (Molecular Devices, CA, USA). To detect whole IgG or IgE, a 96-well plate was coated with anti-mouse Igs (Southern Biotech).

Competitive ELISA
For competitive ELISA, we used biotin-conjugated anti-HA IgG antibodies purified from a hybridoma (35), along with HRP-conjugated streptavidin as previously reports (14). The titer was determined by the inhibition curve based on the absorbance of standard anti-HA IgG antibody.

Antibody expression in vivo
We performed hydrodynamic injections (38, 39) using a previously described method (14). Briefly, six to ten week-old Balb/c mice were injected in the tail vein with PBS-containing plasmid (e.g. 5 µg/1.6 ml), where the DNA volume was 8–12% of the body weight. The injection was performed over less than 5 s using a 27-gauge needle. One day later, the serum was obtained and treated with RDE (II) overnight at 37°C, followed by the measurement of the expression level of anti-HA IgE and anti-HA IgG or whole IgE and IgG by quantitative ELISA.

Western blotting and Coomassie Brilliant Blue (CBB) staining
Anti-HA IgE and anti-HA IgG in the supernatants, or purified IgE (C38-2) and IgG (15H6) treated with RDE (II) were separated by SDS-PAGE (6% or 12%) under non-reducing or reducing conditions, followed by either transfer to a PVDF membrane (Immobilon-P) (Merck Millipore) or stained with CBB (BioRad, Hercules, CA, USA). The membrane was blocked with Blocking One reagent (Nacalai Tesque) for 30 min, followed by incubation at room temperature with a mixture of HRP-conjugated goat anti-mouse IgG and IgE or light chain (kappa) BP (Santa Cruz, Dallas, TX, USA). The specific bands were visualized using the enhanced chemiluminescence ECL substrate (GE Healthcare) on the ImageQuant LAS4000 system (GE Healthcare).

Bone marrow-derived mast cells (BMMCs) culture and stimulation
BMMCs were generated from Balb/c mice, as previously described (40, 41). Briefly, mouse bone marrow cells were harvested from the tibias and femurs, and cultured for 4-6 weeks in RPMI1640 (Thermo Fisher Scientific)
containing IL-3 obtained from IL-3-expressing CHO cells, 10% fetal calf serum, 1 mM pyruvic acid (Wako), 0.1 mM non-essential amino acids (Wako), 50 µM 2-mercaptoethanol, and penicillin–streptomycin–glutamine. Anti-DNP IgE, which was kindly provided by Dr. Fu-Tong Liu (University of California) (9), was treated with RDE (II) overnight (12-20 h), followed by incubation at 56°C for 30 min. Then, BMMCs were incubated with the RDE (II)-treated anti-DNP IgE for 2 h or overnight (12-20 h), followed by incubation with HSA-DNP (Sigma-Aldrich) as indicated either overnight (12-20 h) or for 1 h. The supernatant was then collected and analyzed using a quantitative ELISA kit for IL-6 (ebioscience), TNF-α, and histamine (Neogen Corp. Lexington, KY, USA).

Flow cytometry to detect the binding of IgE to BMMCs

Twenty µg/ml biotin-conjugated anti-DNP IgE or mouse IgG1 isotype control antibody (MOPC-21; Biolegend, San Diego, CA, USA) was treated with RDE (II) overnight (12–20 h). BMMCs or bone marrow-derived macrophages (BMMs), which differentiated in the presence of L929 cells-conditioned medium as mentioned in a previous report (42), were incubated with the antibodies for 30 min at 4°C, followed by incubation with APC-conjugated streptavidin (Biolegend). The binding level was analyzed by FACSCantoII (BD Bioscience, Franklin Lakes, NJ, USA).

IgE-mediated passive cutaneous anaphylaxis (PCA)

We performed mast cell-dependent PCA experiments, which is known as a model of in vivo type I allergy, as previously described (43–45). Briefly, six to ten week-old Balb/c mice were primed with 20 ng/10 µl RDE (II)-treated anti-DNP IgE or untreated at both ears under pentobarbital anesthesia (Somnopentyl, Kyoritsu, Tokyo, Japan). One day later, the mice were intravenously injected with 200 µg HSA-DNP in 200 µl of PBS(−)-containing 0.5% Evans blue dye (Sigma) to visualize IgE-mediated anaphylaxis, by which vascular permeability increased. Thirty minutes later, an 8-mm ear punch was collected and minced in 50 µl of 1 N potassium hydroxide and incubated at 37°C overnight. To extract the Evand’s blue dye, 260 µl of extraction buffer (0.4 N Phosphoritic acid and 72% (v/v) acetone) was added to the specimen. The absorbance was determined at 620 nm by Spectramax M5.

Lectin array

Lectin conjugate microarray production and analysis were performed as described previously (19). Briefly, biotin-conjugated IgE (anti-TNP IgE (C38-2) and anti-DNP IgE) were concentrated with DynabeadsM280 Streptavidin (ThermoFisher Scientific) and dissolved in the Matsunami spotting solution. After filtration, they were spotted on the Schott epoxy-coated glass slide using the MicroSys non-contact microarray printing robot. Cy3-labeled streptavidin dissolved in the probing solution were applied to each chamber of the lectin conjugate microarray (80 µl/well) and were incubated at 20°C overnight. After washing the
RDE modulates IgE to not induce anaphylaxis

Chambers with the probing solution, fluorescent images were immediately acquired using a Bio-Rex scan 200 evanescent-field activated fluorescence scanner (Rexxam Co. Ltd., Kagawa, Japan). Data were analyzed with the Array Pro analyzer version 4.5 (Media Cybernetics, Inc.). The net intensity value for each spot was determined by signal intensity minus background value. The lectin signals of triplicate spots were averaged and normalized to the highest signal intensity among 96 lectin conjugates immobilized on the array.

Lectin blotting
The binding activity of RDE (II)-treated IgE to the lectins was analyzed by lectin blotting as described previously (46). Briefly, purified IgG and IgE were separated by 6% SDS-PAGE, followed by transfer to a PVDF membrane and blocking. Then, the membrane was incubated with biotin conjugated-Lycopersicon esculentum lectin (LEL) (Vector Laboratories, Inc., Burlingame, CA, USA), or Phaseolus vulgaris leucoagglutinin (PHA-L) at room temperature for 2 h, followed by incubation with HRP-conjugated streptavidin (GE Healthcare) for 30 min.

Statistical analysis
All graphs were constructed using GraphPad Prism 7 (GraphPad Software). Data were analyzed using parametric one-way ANOVA or Student’s t-test, where P<0.05 was considered statistically significant. Data are shown and compared as mean and ranges of more than 5 mice in different groups.
RDE modulates IgE to not induce anaphylaxis

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RDE modulates IgE to not induce anaphylaxis

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**FOOTNOTES**

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The abbreviations used are: Ab, antibody; BMM, bone marrow-derived macrophage; BMMC, bone
RDE modulates IgE to not induce anaphylaxis

marrow derived-mast cell; BNPP, bis-p-nitrophenyl phosphate; (light chain) BP, binding protein; CBB, Coomassie Brilliant Blue; DFP, diisopropyl fluorophosphates; DMSO, Dimethyl sulfoxide; DNP, dinitrophenol; Endo F1, endoglycosidase F1; FcεRI, high-affinity fragment crystallizable ε receptor; GlcNAc, N-acetylglucosamine; LacNAc, N-acetyl-lactosamine; RDE, receptor destroying enzyme; HA, hemagglutinin; HAS, human serum albumin; HEK293T, Human Embryonic Kidney cells 293 that stably express the SV40 large T antigen; LEL, Lycopersicon esculentum lectin; NA, neuraminidase (sialidase); PCA, passive cutaneous anaphylaxis; PHA-L, Phaseolus Vulgaris Leucoagglutinin; PI, Propidium Iodide; PNGase F, Peptide-N-glycosidase F; SSC, side scatte: TNP, trinitrophenyl; TNF-α, Tumor Necrosis Factor-α;

Figure legends

Figure 1. RDE (II) inactivates anti-HA IgE, but not anti-HA IgG. (A) HEK293T cells were transfected with pCADEST1-anti-HA IgG or anti-HA IgE, and anti-HA kappa. One week later, the supernatants were collected and treated with RDE (II) for 6 h. Then, the antigen-binding level of anti-HA IgE and anti-HA IgG was analyzed by competitive ELISA. (B) The expression level of the RDE (II)-treated antibodies in the supernatant was analyzed by quantitative ELISA coated with anti-mouse Ig. (C) The supernatants that were treated with RDE (II) were blotted under non-reducing conditions. They were analyzed with HRP-conjugated light chain BP. Data are representative of at least two independent experiments and indicate the mean ± standard deviation. ***P<0.001 (Student’s t-test).

Figure 2. RDE (II) changes the structure of IgE and reduces its binding activity to anti-ε chain, but not that of IgG. (A) Serially diluted purified IgG (Clone: 15H6) and IgE (Clone: C38-2) (187.5 ng~, 3-fold dilutions) were treated with RDE (II) at 37°C overnight (12-20 h). They were blotted under non-reducing conditions with HRP-conjugated anti-mouse IgG and IgE. (B) Purified IgG and IgE (140 ng) were treated with RDE (II). They were analyzed with CBB staining. (C) Purified IgE and IgG were treated with RDE (II). They were blotted by SDS-PAGE under non-reducing conditions with HRP-conjugated light chain binding protein (BP). (D) Purified IgE treated with diluted RDE (II) (3-fold dilutions, indicated number means multiplier (= n)) was blotted under reducing conditions with HRP-conjugated anti-mouse IgE. (E) Purified IgG and IgE were treated with RDE (II). They were blotted under reducing conditions with HRP-conjugated light chain BP. (F and G) Purified IgE (F) or IgG (G) were treated with diluted RDE (II) as indicated overnight (12-20 h). The level of IgE (F) and IgG (G) were measured by quantitative ELISA. Data are representative of two independent experiments and indicate the mean ± standard deviation.
RDE modulates IgE to not induce anaphylaxis

**Figure 3. RDE (II) inactivates serum IgE antibodies, but not IgG.** (A and B) Balb/c mice were injected with pCADEST1-anti-HA IgG or anti-HA IgE, and pCADEST1-anti-HA kappa by hydrodynamic injection. One day later, the serum was obtained and treated with RDE (II) overnight ex vivo. Then, the specimens were incubated at 56°C for 30 min. Anti-HA IgE and IgG level was analyzed by quantitative ELISA. (C and D) The serum, which was obtained from naïve mice, was treated with RDE (II) overnight (12-20 h). Total IgE (C) and IgG (D) levels were analyzed by quantitative ELISA. Data are representative of two independent experiments and indicate the mean ± standard deviation. ***P<0.001 (Student’s t-test).

**Figure 4. RDE (II)-treated IgE cannot induce anaphylaxis.** (A and B) Effects of RDE (II)-treated IgE were reducing the expression level of IL-6 (A) or TNF-α (B) from BMMCs. Two µg/ml anti-DNP IgE was treated with RDE (II) overnight (12-20 h), followed by incubation at 56°C. Then, the antibodies sensed BMMC for 2 h, followed by incubation with HSA-DNP as indicated overnight (12-20 h). After, the supernatant was collected and analyzed with quantitative ELISA for IL-6 (A) or TNF-α (B). **P<0.01, ***P<0.001 (Student’s t-test). (C) Two µg/ml anti-DNP IgE were treated with RDE (II) overnight (12-20 h). BMMCs were sensed overnight (12-20 h) then incubated with 200 ng/ml HSA-DNP for 1 h. The supernatant was then obtained and analyzed with competitive ELISA for histamine. ***P<0.001 (one-way ANOVA). (D) Twenty µg/ml biotin-conjugated anti-DNP IgE or control IgG (MOPC-21) was treated with RDE (II) overnight (12-20 h). BMMCs or BMMs were incubated with the antibodies for 30 min, followed by incubation with APC-conjugated streptavidin. The binding level was analyzed by FACSCantoII. (E) Anti-DNP IgE was treated with RDE (II) overnight (12-20 h). The ears of mice were passively sensitized with 20 ng RDE (II)-treated anti-DNP IgE. One day later (18-24 h), all mice were intravenously injected with 200 µg DNP-HSA containing Evans blue dye. Thirty minutes later, the ears were obtained and observed in the transudation of Evans blue dye to determine vascular permeability. (F) Punched with 8 mm piece from the ears for extraction of the Evans blue dye. The optical density was measured at 620 nm. ***P<0.001 (one-way ANOVA) All data are representative of at least two independent experiments and indicate the mean ± standard deviation.

**Figure 5. Characteristics of RDE (II) for modulating IgE.** (A-B) RDE (II) was pre-incubated at 56°C or 100°C for 30 min. Purified IgE was treated with the RDE (II) overnight (12-20 h). The level of IgE was measured by quantitative ELISA (A), and they were also blotted and analyzed with HRP-conjugated goat mouse anti-IgE (B). (C) Purified IgE was treated with dialyzed RDE (II) overnight (12-20 h). They were blotted by western blotting under non-reducing conditions. (D) Purified IgE were treated with RDE (II) for the indicated time. They were blotted under non-reducing conditions. Data are representative of two independent experiments and indicate the mean ± standard deviation. ***P<0.001 (one-way ANOVA).
RDE modulates IgE to not induce anaphylaxis

Figure 6. RDE (II) modulates the glycan on IgE. (A) Lectin microarray data of RDE (II) treated-IgE (#1, #2) and untreated (#1, #2) were mean-normalized and then analyzed by Cluster 3.0. Clustering method was complete linkage. The heat map with clustering was acquired using Java Treeview. Yellow: positive, Blue: negative. (B and C) Indicated data ($P<0.01$) represent the mean-normalized signal intensities of LEL (B) and PHA-L (C). The sugar residue or oligosaccharide structure in a parenthesis represents the binding preference. The glycan in parenthesis represents carbohydrate specificity for each lectin. #1: anti-TNP IgE (C38-2), #2: anti-DNP IgE. (D-G) Relationship between the exercises of RDE (II) and the glycan structures. Purified IgE (C38-2) or IgG (15H6) were treated with the RDE (II) overnight (12-20 h). They were blotted under non-reducing conditions. The specimens were analyzed with biotin-conjugated LEL (D) or PHA-L (F), followed by incubation with HRP-conjugated streptavidin. (E) and (G) are internal control for each (D) and (F), which is detected with HRP-conjugated anti-mouse IgE and IgG. Data (D–G) are representative of two independent experiments.
Yamazaki et al. Figure 2
Figure 3

A. Serum anti-HA IgE

B. Serum anti-HA IgG

C. Serum IgE

D. Serum IgG
**Figure 4**

(A) IL-6 (pg/ml) levels in BMMC treated with different concentrations of DNP (ng/ml) and RDE (II).

(B) TNF-α (pg/ml) levels in BMMC treated with different concentrations of DNP (ng/ml) and RDE (II).

(C) Histamine levels in BMMC treated with DNP and RDE (II).

(D) Flow cytometry analysis of BMMC treated with Anti-DNP IgE and RDE (II) treated-anti-DNP IgE.

(E) Ear skin Evans blue dye leakage (OD$_{620}$) levels with Vehicle, Anti-DNP-IgE, and RDE (II) treated-anti-DNP-IgE.

(F) Bar graph showing % of Max Evans blue dye leakage (OD$_{620}$) in Ear skin with Vehicle, Anti-DNP-IgE, and RDE (II) treated-anti-DNP-IgE.
**Figure 6**

(A) Diagram showing the detected proteins with corresponding molecular weights (kDa) for IgE and IgG under non-reducing conditions with Vehicle and RDE(II) treatments.

(B) Graph showing the mean-normalized signal intensity for IgE and IgG under Vehicle and RDE(II) conditions.

(C) Graph showing the mean-normalized signal intensity for PHA-L under Vehicle and RDE(II) conditions.

(D) Detection by LEL (Non-reducing conditions)

|          | IgE | IgG | Vehicle | RDE(II) |
|----------|-----|-----|---------|---------|
| IgE      | +   | +   | -       | -       |
| IgG      | -   | -   | +       | +       |
| Vehicle  | +   | -   | +       | +       |
| RDE(II)  | -   | +   | -       | +       |

(E) Detection by anti-mouse IgE and IgG (Non-reducing conditions)

|          | IgE | IgG | Vehicle | RDE(II) |
|----------|-----|-----|---------|---------|
| IgE      | +   | +   | -       | -       |
| IgG      | -   | -   | +       | +       |
| Vehicle  | +   | -   | +       | +       |
| RDE(II)  | -   | +   | -       | +       |

(F) Detection by PHA-L (Non-reducing conditions)

|          | IgE | IgG | Vehicle | RDE(II) |
|----------|-----|-----|---------|---------|
| IgE      | +   | +   | -       | -       |
| IgG      | -   | -   | +       | +       |
| Vehicle  | +   | -   | +       | +       |
| RDE(II)  | -   | +   | -       | +       |

(G) Detection by anti-mouse IgE and IgG (Non-reducing conditions)

|          | IgE | IgG | Vehicle | RDE(II) |
|----------|-----|-----|---------|---------|
| IgE      | +   | +   | -       | -       |
| IgG      | -   | -   | +       | +       |
| Vehicle  | +   | -   | +       | +       |
| RDE(II)  | -   | +   | -       | +       |
Receptor destroying enzyme (RDE) from Vibrio cholerae modulates IgE activity and reduces the initiation of anaphylaxis

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