

A Screening Method for Antigen-Specific IgE Using Mast Cells Based on Intracellular Calcium Signaling

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A simple screening method is presented for the measurement of antigen-specific IgEs in sera in which mast cells are used. This method is based on the intracellular calcium signal in mast cells induced by cross-linking the surface high-affinity Fc receptors (FcεR1s) with IgEs and multivalent antigens. When a serum containing various antigen-specific IgEs is added to the mast cell suspension, various antigen-specific IgEs are captured by FcεR1s on the cell surface. However, the required antigen-specific IgE can be specifically detected after the addition of the corresponding antigen. The resulting increase in intracellular calcium concentration ($[Ca^{2+}]_i$), monitored by Ca^{2+} -fluorometry, was found to be an analytical measure for the screening of IgEs. Two kinds of rodent mast cells, cell-lined RBL-2H3 cells and primary cultured BMMCs, were used as a representative model system of mast cells. A DNP hapten (DNP₃₅-HSA) and ovalbumin (OVA) were chosen for illustrative antigens, and these antigen-specific IgEs (DNP-specific IgE, OVA-specific IgE) in the corresponding rodent sera were target antibodies. It was found that $[Ca^{2+}]_i$ increased linearly with IgE concentrations ranging from 25 to 5000 ng/mL for DNP-specific IgE and from 5 to 50 ng/mL for OVA-specific IgE. For these dynamic ranges, optimum concentrations of antigens were found to be 10 ng/mL and 1 μg/mL for DNP₃₅-HSA and OVA, respectively. It was concluded that by monitoring the increase of $[Ca^{2+}]_i$ in mast cells, we could determine the antigen-specific IgEs. The present immunological assay based on the Ca^{2+} signal transduction in mast cells offers new possibilities for efficient screening of antigen-specific IgEs and the immunogenicity of IgE in sera.

Immunoglobulin Es (IgE) constitutes a minuscule fraction of the total antibodies in human and animal sera. However, its action is powerfully amplified by the activities of the Fc receptor to which it binds. The high-affinity Fc receptor (FcεRI) ($K_a = 10^{10} M^{-1}$) on mast cells and basophils is responsible for immediate hypersensitivity.

IgE is produced in mammalian blood against various types of antigens, and it causes typical immediate-type hypersensitivity like asthma, atopic dermatitis, and food allergies. Dust mites, molds, and tree pollens are known to be major allergens for asthma. Ovalbumin, milk, and soybeans are major allergens for food allergies. Furthermore, penicillin or other small compounds (e.g., trimellitic anhydride or toluene diisocyanate) are shown to produce IgEs and cause anaphylaxis.

The determination of antigen-specific IgEs is an important task required in the diagnosis of immediate-type hypersensitivity. The passive cutaneous anaphylaxis (PCA) assay¹ and the enzyme-linked immunosorbent assay (ELISA) have been the standard methods used to evaluate the concentration of IgEs. However, the PCA assay and ELISA suffer from the following problems. In the PCA assay, the antibody concentration is determined as the greatest dilution that yields a positive result (Evan's Blue) upon examination of the underside of the skin. The PCA assay, as an in vivo bioassay, is subject to individual animal variability. In addition, many animals have to be sacrificed to obtain reliable data and therefore it is very expensive when it is used. ELISA would be an obvious alternative to the PCA assay. ELISA has become widely accepted as a useful method to detect and quantify IgEs because of simplicity and cost. Although ELISA can quantify the concentration of antigen-specific IgEs, the competition of the same antigen-specific IgG (Immunoglobulin G) cannot be neglected. This is attributed to a very small percentage of IgE antibodies relative to IgGs in sera, and because of the competitive binding inhibition of antigen-specific IgE antibodies to antigen-coated plates by antigen-specific IgG antibodies, IgE concentrations are sometimes estimated lower than the real ones.

Mast cells, such as rat basophilic leukemia (RBL) cells^{2,3} and bone-marrow-derived mast cells^{4,5} (BMMCs), bear FcεR1s on the cell surface, and the Fc portion of IgE can be specifically captured

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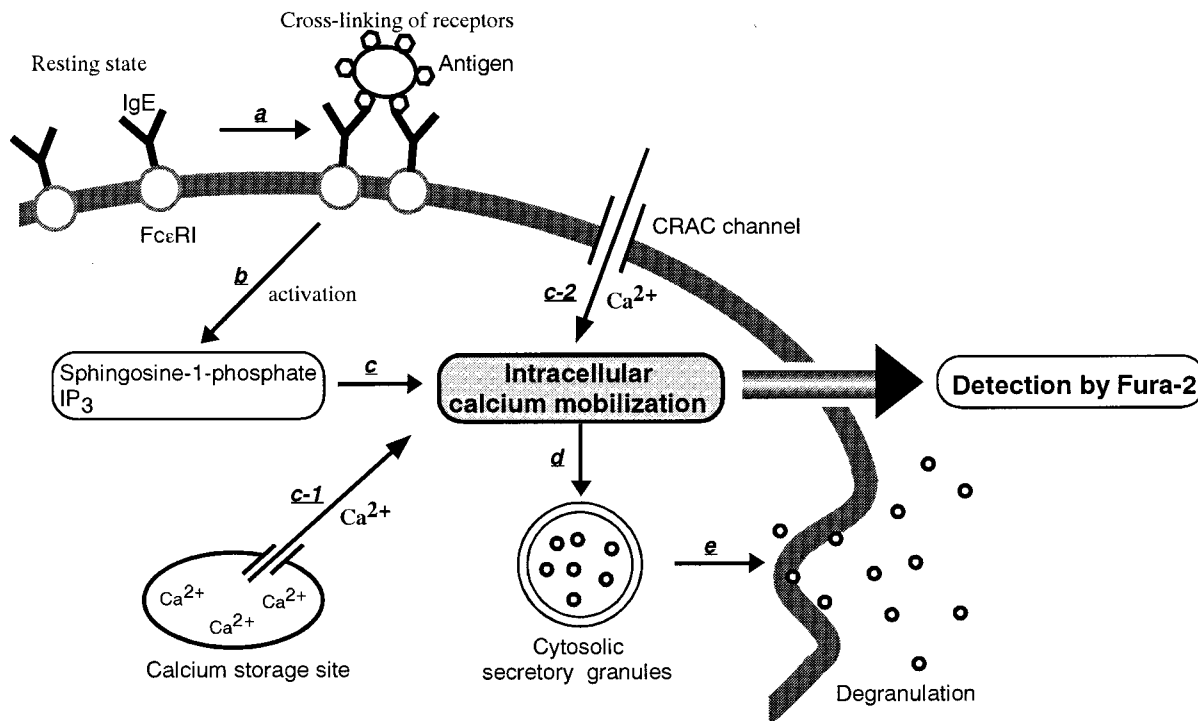


Figure 1. Illustration of the intracellular signal transduction in mast cells.

by this receptor.⁶ FcεRIs are expressed primarily by mast cells and basophils, and these cells play a critical role in immediate-type hypersensitivity. Figure 1 shows the signal transduction scheme in mast cells. When a particular multivalent antigen associates with two neighboring IgE-bound FcεRI units, it cross-links these two complexes on the cell membrane (a). The importance of this receptor cross-linking has been widely recognized⁷ as the first step for the intracellular signal transduction. The resulting phosphorylation of FcεRI initiates an increase in concentrations of sphingosine 1-phosphate⁸ and IP₃.^{9,10}

Sphingosine 1-phosphate and IP₃ trigger the calcium signal (b). This calcium ion mobilization is one of the earliest and most important events in the intracellular signal transduction in mast cells. The release of Ca²⁺ from the intracellular calcium storage site (i.e., endoplasmic reticulum) is generated (c-1), and the influx of Ca²⁺ through the calcium-release-activated calcium (CRAC) channel is induced (c-2). The sustained increase in [Ca²⁺]_i (c) promotes the release into the extracellular space (d, e) of mediators for immediate hypersensitivity¹¹ from cytoplasmic secretory granules upon its degranulation. Such a sustained intracellular Ca²⁺ mobilization is indispensable for this degranulation.^{11,12} Of such mediators, histamine, β-hexosaminidase, and serotonin contained in the secretory granules are important in eliciting symptoms associated with allergic reactions.

In the present study, we propose a new, convenient, and time-saving screening method for the determination of IgEs in a serum

based on intracellular Ca²⁺ signal transduction in mast cells. Using cell-lined RBL-2H3 cells and primary cultured mouse BMMCs, we found that [Ca²⁺]_i can be a 1:1 probe for the concentration of IgE in a mouse serum sample. The principle of the proposed method for the screening of the antigen-specific IgE in the serum is schematically shown in Figure 2. Upon addition of the serum containing various antigen-specific IgEs to the cell suspension, these antigen-specific IgEs are captured by a single FcεRI on the cell surface. When the screening of OVA-specific IgE in the serum is required, for example, the ovalbumin (OVA)-specific IgE bound to FcεRI can be selectively picked up by adding its antigen OVA to cross-link the two neighboring identical receptor-antibody complexes on the cell surface. The intracellular Ca²⁺ signal is thereby provoked, which is finally detected as an analytical measure for the target IgE level in the sample serum. Although various antigen-specific IgEs are generally contained in a serum, one desired antigen-specific IgE can thus specifically be detected by the present method.

The very small percentage in a serum of the target IgE in comparison with its counterpart IgG does not prevent the measurement of the IgE in the proposed assay because FcεRI can specifically bind to IgE, and it is one of the important advantages over ELISA. Because ELISA is a binding assay, the antigen-specific IgE in a serum binds to the antigen adsorbed on a plate regardless of whether the antigen is multivalent or not. However, in mast cells, the monovalent antigen without cross-linking of receptors cannot induce the intracellular signal transduction. Only a specific IgE that evokes hypersensitivity can thus be determined.

EXPERIMENTAL SECTION

Reagents. A fluorescent dye reagent for the calcium ion, Fura-2/AM, was obtained from Dojindo (Kumamoto, Japan). Dinitrophenylated human serum albumin (DNP₃₅-HSA) and chicken egg

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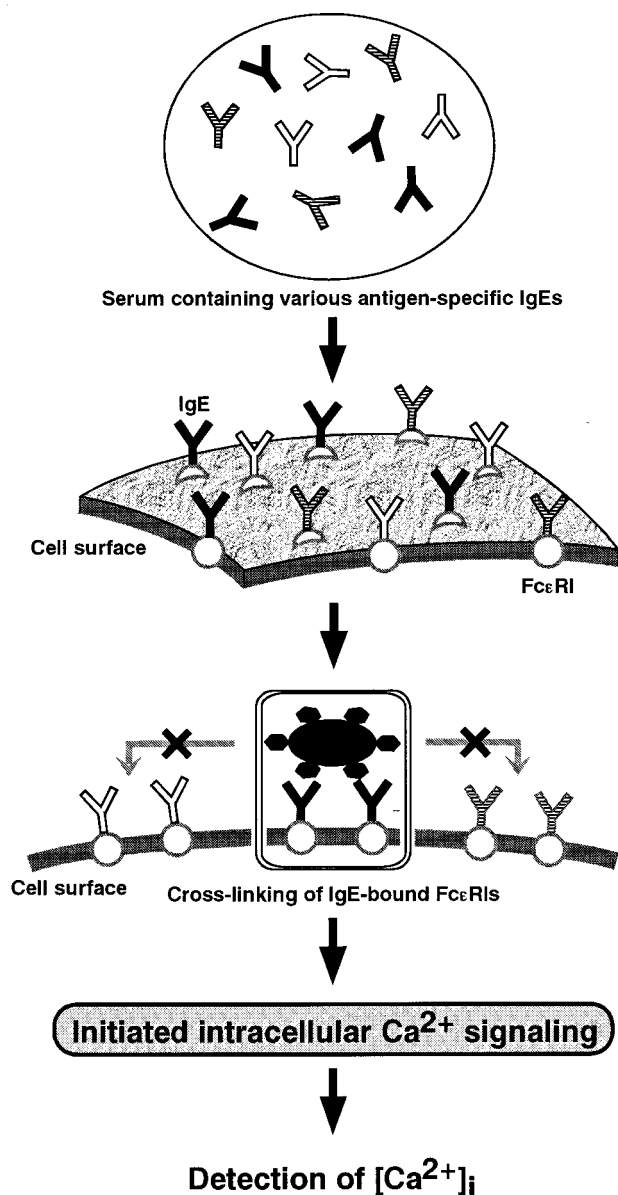


Figure 2. Principle of the present method based on the signal transduction in mast cells.

albumin (OVA) were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui (Tokyo), fetal bovine serum from Dainippon Seiyaku (Osaka, Japan), and PIPES from Wako Pure Chemical Industries (Osaka, Japan).

Cells. RBL-2H3 cells¹³ were maintained in DMEM supplemented with 10% (v/v) fetal calf serum. BMMCs^{4,5} were obtained from the bone marrow of 10-week-old female BALB/c mice (SLC, Hamamatsu, Japan) and maintained in DMEM supplemented with 10% (v/v) fetal calf serum, 0.1 mM nonessential amino acids, antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), IL-3 (50 U/mL), and 50% WEHI-culture supernate, as described previously.⁵

Antibodies. Mouse anti-dinitrophenyl (DNP) IgE monoclonal antibodies were prepared as described previously.¹⁴ An OVA-specific serum was generated in female BALB/c mice by the

method of Kawabata et al.,¹⁵ and the ELISA titer of the obtained OVA-specific IgE was determined by the method described below.

ELISA for the Mouse Serum OVA-Specific IgE Titer. The mouse serum titer of OVA-specific IgE was determined by the method of Teshima et al. with some modifications.¹⁶ Fifty microliters of OVA (40 μ g/mL) in 50 mM sodium carbonate buffer, pH 9.6, was added to each well of a 96-well microtiter plate and incubated overnight at 4 °C. The solution was discarded, and each well was washed four times with 200 μ L of PBS containing 0.05% Tween 20 (PBS/Tween). To minimize nonspecific binding of serum proteins to unoccupied solid-phase sites, 200 μ L of 0.1% casein in PBS was added and the plates were incubated for 1 h at room temperature. The casein solution was removed, and each well was washed in the same way as above. Fifty microliters of the diluted serum for OVA-specific IgE was added to each well, and the plates were incubated for 20 h at 4 °C. The solution was removed, and each well was washed. Fifty microliters of rabbit anti-mouse IgE (10^3 -fold dilution in PBS containing 0.1% casein (Yamasa Shoyu, Chiba, Japan)) was added to each well, and the plates were incubated for 1 h at room temperature. The solution in each well was removed and washed. Fifty microliters of β -galactosidase-linked goat anti-rabbit Ig conjugate (10^3 -fold dilution in PBS containing 0.1% casein) was added to each well, and the plates were incubated for 1 h at room temperature. The antibody-enzyme conjugate solution in each well was removed and washed. The wells were incubated for 1 h at 37 °C with 100 μ L of PBS containing 0.1 mM 4-methylumbelliferone- β -galactoside (Sigma, St. Louis, MO). Finally, 25 μ L of 1 M anhydrous sodium carbonate was added to each well. The fluorescence intensity of the liberated 4-methylumbelliferone was monitored at 317 and 374 nm for excitation and emission, respectively, by a Titertek Fluoroscan reader (Flow Laboratories, Costa Mesa, CA).

Measurement of the Cytosolic Free Calcium Concentration ($[Ca^{2+}]_i$). The buffer used had the following composition: 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM glucose, and 10 mM PIPES, pH 7.4.

RBL-2H3 cells (6×10^5 cells/mL) or BMMCs (4×10^5 cells/mL) were sensitized (i.e., preincubated) with various concentrations of DNP-specific IgE (0.5–5000 ng/mL for RBL-2H3 cells and 1 ng/mL–10 μ g/mL for BMMCs) or OVA-specific IgE (5–50 ng/mL for RBL-2H3 cells and 5 ng/mL–50 ng/mL for BMMCs) and loaded with 6 μ M fura-2/AM. In the case of loading OVA-specific IgE, 0.05% Pluronic F-127 was added with Fura-2/AM.¹⁷ Pluronic F-127 is a nonionic dispersing agent that helps to solubilize dye molecules in physiological media. After removal of the free dye and unbound IgE by centrifugation, the cells were resuspended in 2 mL of PIPES buffer and stimulated with DNP-HSA (10^{-2} –100 ng/mL for RBL-2H3 cells and 1 ng/mL–100 μ g/mL for BMMCs) or OVA (1 ng/mL–10 μ g/mL for RBL-2H3 cells and 100 ng/mL–10 μ g/mL for BMMCs). Fluorescence measurements were made in a 1-cm quartz cuvette using a Shimadzu RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). Fura-

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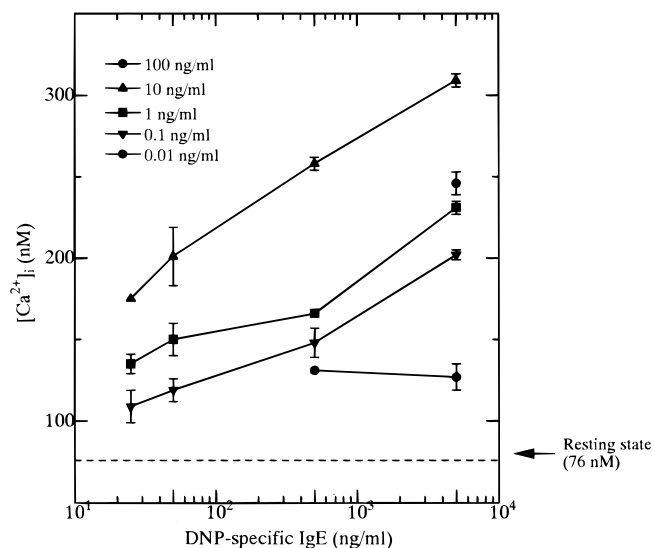


Figure 3. Calcium response induced by stimulating with the antigen (DNP-HSA) after sensitization with DNP-specific IgE in RBL-2H3 cells. Each value represents the mean $\pm$ SD of three to seven experiments.

2-loaded cells were alternatively excited at 335 and 362 nm,¹⁸ and the resulting fluorescence emissions before and after stimulation with a given antigen for the cross-linking of the IgE-receptor complexes were observed at 495 nm; the intracellular calcium ion concentration was calculated therefrom by the method of Tsien et al.¹⁹ The temperature of the observation chamber was kept at 37 °C.

RESULTS AND DISCUSSION

The proposed method was tested using representative model systems to determine whether the intracellular calcium ion concentration in mast cells can in fact be a 1:1 probe for the level of IgE concentrations in a serum and be applicable for the screening of the antigen-specific IgE. We measured the free calcium ion concentration ($[Ca^{2+}]_i$) in two kinds of rodent mast cells (RBL-2H3 cells and BMMCs), which were stimulated with various concentrations of antigens (DNP-HSA and OVA) after sensitization with antigen-specific mouse IgEs (DNP-specific IgE and OVA-specific IgE). The DNP hapten and OVA are typical well-studied antigens, and their antigen determinants are well-known. Therefore, DNP-specific IgE and OVA-specific IgE were used as representative examples for the analyte IgEs.

Ca^{2+} Response Induced by IgE and Antigen. a. Ca^{2+} Response in RBL-2H3 Cells. The cells were sensitized with various concentrations of the IgE (25 ng/mL–5 μ g/mL) and then stimulated with various concentrations of antigen (DNP-HSA) (10⁻²–100 ng/mL). The $[Ca^{2+}]_i$ level in the resting cells in this case was 64–86 nM. The dependence of $[Ca^{2+}]_i$ in RBL-2H3 cells on the concentrations of the DNP-specific IgE and the antigen (DNP-HSA) is shown in Figure 3. The largest Ca^{2+} response was observed when cells were sensitized with 5 μ g/mL DNP-specific IgE and stimulated with 10 ng/mL antigen. The minimum detectable concentration of DNP-specific IgE in RBL-2H3 cells was

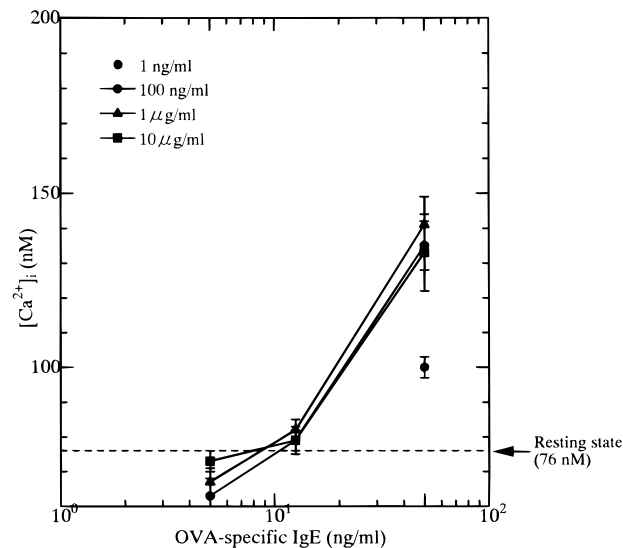


Figure 4. Calcium response induced by stimulating with the antigen (OVA) after sensitization with OVA-specific IgE in RBL-2H3 cells. Each value represents the mean $\pm$ SD of three to four experiments.

<25 ng/mL, when cells were stimulated with 10 ng/mL DNP-HSA. The increase of $[Ca^{2+}]_i$ induced by stimulation with 10 ng/mL DNP-HSA was dependent on the increase in the amount of DNP-specific IgE that was used for the cell sensitization. $[Ca^{2+}]_i$ increased from 150 to 300 nM in response to the increase in the IgE concentration from 25 ng/mL to 5 μ g/mL.

The cells were stimulated with various concentrations of antigen (OVA) (100 ng/mL–10 μ g/mL) after sensitization with various concentrations of OVA-specific IgE (5–50 ng/mL), and the optimum concentration of OVA for the measurement of the OVA-specific IgE was determined. The $[Ca^{2+}]_i$ level in the resting cells in this case was 70–80 nM. The dependence of $[Ca^{2+}]_i$ in RBL-2H3 cells on the concentration of the OVA-specific IgE and the antigen (OVA) is shown in Figure 4. The largest $[Ca^{2+}]_i$ was observed when cells were stimulated with the antigen at 0.1 or 1 μ g/mL, and the optimum concentration of OVA to measure the OVA-specific IgE was determined to be 0.1 or 1 μ g/mL. When the antigen concentration was lowered to 1 ng/mL, the increase in $[Ca^{2+}]_i$ was substantially reduced (data not shown). The antigen at <1 ng/mL was incapable of further mobilizing the $[Ca^{2+}]_i$ increase. The detection limit of OVA-specific IgE was <25 ng/mL, when RBL-2H3 cells were stimulated with OVA at 1 μ g/mL.

Concentrations of 25 ng/mL DNP-specific IgE and 25 ng/mL OVA-specific IgE were thus found to be detectable in the present IgE-receptor cross-linking and $[Ca^{2+}]_i$ -monitoring method using RBL-2H3 cells.

The obtained maximum $[Ca^{2+}]_i$ response in RBL-2H3 cells sensitized with OVA-specific IgE (Figure 4) was compared with that in the cells sensitized with DNP-specific IgE (Figure 3); the former $[Ca^{2+}]_i$ (140 nM when 50 ng/mL OVA-specific IgE was used) was lower than the latter $[Ca^{2+}]_i$ (200 nM when 50 ng/mL DNP-specific IgE was used). The effect on $[Ca^{2+}]_i$ of the dispersing agent Pluronic F-127 loaded into the cells with OVA-specific IgE cannot be neglected. The correlation between $[Ca^{2+}]_i$ and the number of antigen determinants was discussed by Torigoe and

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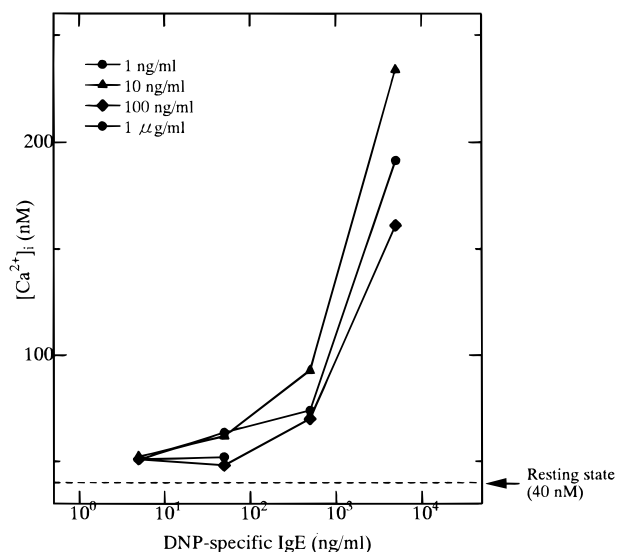


Figure 5. Calcium response induced by stimulating with the antigen (DNP-HSA) after sensitization with DNP-specific IgE in BMMCs. Similar results were obtained in the other two experiments.

Nakanishi,²⁰ who showed that the adequate number of antigen determinants on the surface of a given antigen molecule is responsible for a larger response of $[Ca^{2+}]_i$. The difference in the Ca^{2+} response shown in the above results may therefore be attributed to a difference in the number of antigen determinants on the surfaces of the respective antigen molecules.

b. Ca^{2+} Response in BMMCs. Though mouse BMMCs are known to easily differentiate from mouse bone-marrow progenitor cells to mast cells within 3 weeks in the presence of IL-3 and culture stably, the Ca^{2+} response in IgE-sensitized and antigen-stimulated BMMCs has not been fully examined. In addition, BMMCs are not cultured cells as RBL-2H3 cells but are primary cultured cells. We examined the Ca^{2+} response of BMMCs and the applicability of BMMCs for the measurement of the IgE concentration. Shown in Figures 5 and 6 are the dependences of $[Ca^{2+}]_i$ in BMMCs on the concentrations of IgEs and antigens.

The cells were sensitized with various concentrations of DNP-specific IgE (5–5000 ng/mL) and stimulated with various concentrations of DNP-HSA (1 ng/mL–1 μ g/mL). The $[Ca^{2+}]_i$ level in the resting cells in this system was 40–45 nM. As shown in Figure 5, the largest $[Ca^{2+}]_i$ was observed when the cells were stimulated with 10 ng/mL DNP-HSA; the optimum concentration of DNP-HSA to measure the IgE concentration was therefore found to be 10 ng/mL. In several sets of the experiments, we obtained similar optimum concentrations of the antigen. When the antigen concentration was lowered to 0.01 ng/mL, the increase in $[Ca^{2+}]_i$ was substantially reduced. The antigen at <0.01 ng/mL could not further mobilize the $[Ca^{2+}]_i$ increase (data not shown). The minimum detectable concentration of DNP-specific IgE in BMMCs was 50 ng/mL, when these cells were stimulated with DNP-HSA at 10 ng/mL. The increase in $[Ca^{2+}]_i$ induced by stimulation with 10 ng/mL DNP-HSA was dependent on the increase in the amount of DNP-specific IgE that was used for the sensitization of BMMCs. $[Ca^{2+}]_i$ increased from 50 to 250 nM in response to an increase in the IgE concentration from 5 ng/mL to 1 μ g/mL.

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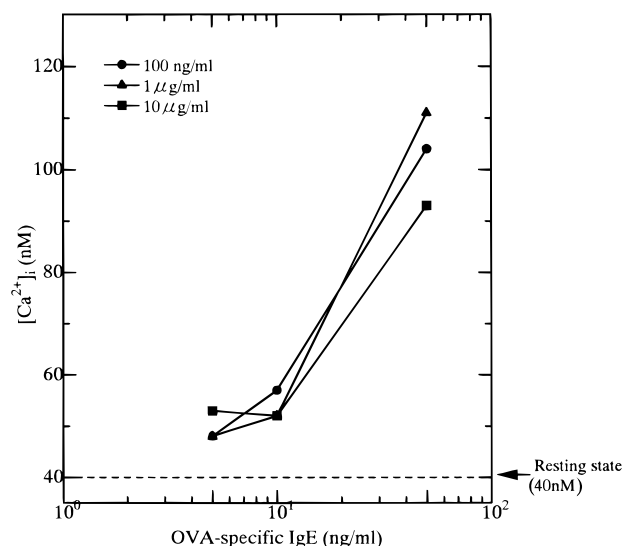


Figure 6. Calcium response induced by stimulating with the antigen (OVA) after sensitization with OVA-specific IgE in BMMCs. Similar results were obtained in the other two experiments.

Next, BMMCs were sensitized with various concentrations of OVA-specific IgE (5–50 ng/mL) and stimulated with various concentrations of OVA (100 ng/mL–10 μ g/mL). When cells were stimulated with the antigen OVA at 10 ng/mL–1 μ g/mL, the dependence of $[Ca^{2+}]_i$ on the antigen concentration was observed as shown in Figure 6. When BMMCs were stimulated with OVA concentrations greater than 10 μ g/mL, $[Ca^{2+}]_i$ was found to level off and decrease (data not shown). From these results, it was concluded that the optimum concentration of this antigen for measurement of the IgE was 1 μ g/mL. The minimum detectable concentration of OVA-specific IgE in BMMCs was 10 ng/mL, when cells were stimulated with OVA at 1 μ g/mL. $[Ca^{2+}]_i$ was found to increase from 50 to 110 nM with the increase in IgE concentration from 5 to 50 ng/mL.

It is worthwhile noting that the proposed method can easily be adapted to any antigen-specific IgE, requiring only the corresponding antigens to cross-link the neighboring two IgE–Fc ϵ RI complexes. Further investigations will therefore be directed toward the optimization of the present method to obtain maximum sensitivity to the IgE concentrations.

Signal transduction events of human mast cells have been poorly examined because of the difficulty in the usage of stable cell lines. Recently, however, stable human mast cells were differentiated from cord blood progenitor cells and their $[Ca^{2+}]_i$ was found to increase upon cross-linking of Fc ϵ RI with anti-IgE antibodies.^{21,22} The mobilization of $[Ca^{2+}]_i$ was found to be important for degranulation in human mast cells, and many similarities were found between the intracellular signal transduction in human mast cells and that in rodent mast cells.²¹ If $[Ca^{2+}]_i$ in human mast cells is found to increase linearly with IgE concentrations for human IgEs, the present method will likewise be applicable to the human systems.

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Significance of the Present Method. IgEs are highly responsible for hypersensitivity, and atopic allergies afflict at least 20% of the populations in developed countries. Atopic allergies fall into a wide range of IgE-mediated disorders, such as asthma, atopic dermatitis, and food allergies, and are generally caused by the overproduction of IgE antibodies in response to common environmental antigens. Some rational in vitro time-saving screening of IgEs is strongly required in the diagnosis of IgE-mediated disorders.

Using the present method, the antigen-specific mouse IgE can be specifically determined from the ubiquitous intracellular Ca^{2+} signaling in intact mast cells, and the reaction between antigens and antibodies is reproduced in this system. The detection limit of this method for the IgE concentration in the examined representative rodent serum samples was 25 ng/mL in the cases of both DNP-specific IgE and OVA-specific IgE, while ELISA gave a detection limit of around 10 ng/mL for the same sample. The detection limit of the present assay is therefore comparable to that of ELISA for the same given samples. The key features of the present method are that neither IgGs nor monovalent IgEs induce the response of the Ca^{2+} signaling and interfere with the assay of the target IgEs.

CONCLUSION

A new, convenient, and time-saving screening method was developed for the determination of IgEs in serum using the intracellular calcium concentration as a probe based on the Ca^{2+} signal transduction in mast cells. As far as we know, this is the

first example of an immunological assay that is based on the intracellular Ca^{2+} signaling and not solely on the antigen–antibody binding. In the present study, two kinds of rodent mast cells, cell-lined RBL-2H3 cells and primary cultured BMMCs, were used as models. The dynamic range for the measurement of the concentration of mouse DNP-specific IgE was 25–5000 ng/mL and that for OVA-specific IgE was 5–50 ng/mL. These dynamic ranges were similar for both types of cells examined. This method may also be applicable to the detection of the production of antigen-specific IgE in the sera of mice immunized with various other antigens for preclinical experiments. It is anticipated that, with the use of primary cultured human mast cells, the present method may readily be applied to the detection of antigen-specific human IgEs and the diagnosis of immediate-type hypersensitivity.

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