

Micro-assay to Measure the Allergenicity of a Kunitz-type Soybean Trypsin Inhibitor toward Balb/c Mice by Using RBL-2H3 Cells

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The allergenicity of a Kunitz-type soybean trypsin inhibitor (KSTI) was investigated by a micro-assay of β -N-acetylhexosaminidase released from RBL-2H3 cells primed with the anti-KSTI serum. KSTI stimulated the release of β -N-acetylhexosaminidase from RBL-2H3 cells primed with the antiserum. The response of RBL-2H3 cells to the reagenic activity of the mouse anti-KSTI serum correlates fairly well with that by the passive cutaneous anaphylaxis (PCA) test, the sensitivity of both assays appearing to be similar. These results suggest that measuring the β -N-acetylhexosaminidase released from RBL-2H3 is a convenient way for studying the allergen or the reagenic activity of a murine serum in place of the PCA test.

The Kunitz-type trypsin inhibitor (KSTI) is a major component of the 2S globulin fraction of soyproteins and is the predominant experimental allergen among soyproteins for Balb/c mice.¹⁾ When the extracted whole soybean proteins were subcutaneously injected with aluminium hydroxide into Balb/c mice, IgE recognizing KSTI would be preferentially produced. Thus, KSTI-sensitive Balb/c mice can be freely prepared as a model to elucidate the mechanism for IgE production. On the other hand, the allergenicity of KSTI toward humans has been reported,^{2–4)} although it might only be a minor dietary allergen for human beings.

The reagenic activity of an animal serum is currently investigated by a passive cutaneous anaphylaxis (PCA) test,⁵⁾ and the allergenicity of KSTI toward Balb/c mice has also been demonstrated by the PCA test. The PCA test involves an *in vivo* method to evoke a local allergic reaction in the subject animal by the intracutaneous injection of an allergic serum and subsequent intravenous injection of the allergen.

It has recently been shown that RBL-2H3, a rat basophilic leukemia cell line, expressed high affinity receptor for IgE (Fc ϵ RI) and that the reagin-primed RBL-2H3 cells could release chemical mediators in response to stimulation of the allergen.⁶⁾ As RBL-2H3 is such a useful tool for studying the secretory response of the basophil *in vitro*, it has been applied in many experiments mainly to analyze the intracellular signal transduction.^{7–10)}

In the present study, we investigated the reagenic activity of the Balb/c mice anti-KSTI serum by measuring the activity of the enzyme, β -N-acetylhexosaminidase (EC 3.2.1.52), released from RBL-2H3 cells and we discuss the suitability of applying this *in vitro* assay in place of the *in vivo* PCA test.

Materials and Methods

Materials. The materials used in the present study were obtained from the sources indicated: RBL-2H3 cells from the Cancer Cell Repository (CCR), Tohoku University (Sendai, Japan); Balb/c mice and Wistar rats from SLC Japan (Shizuoka, Japan); KSTI, 2,4-dinitrophenol (DNP)-

conjugated human serum albumin (HSA) and anti-DNP IgE mouse monoclonal antibody (mAb) from Sigma (St. Louis, MO, U.S.A.); minimum essential medium (MEM), penicillin-streptomycin, trypsin plus ethylenediamine-tetraacetic acid (EDTA) and L-glutamate from GIBCO BRL (NY, U.S.A.); fetal calf serum from Irvine Scientific (St. Ana, CA, U.S.A.); *p*-nitrophenyl-N-acetyl- β -D-glucosaminide from Seikagaku Corporation (Tokyo, Japan); and nembutal from Abbott (North Chicago, IL, U.S.A.).

Cell culture. RBL-2H3 cells were maintained in MEM supplemented with 10% heat-inactivated FCS, 4 mM L-glutamate, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The cells were harvested by treating with 0.25% trypsin and 0.1 mM EDTA, and recovered by centrifugation (250 $\times$ g for 5 min at room temperature).

Immunization of the mice. Balb/c mice were subcutaneously injected four times at two-weeks intervals with 0.3 ml of the immunogen solution containing 0.2 mg/ml of KSTI and 15 mg/ml of aluminium hydroxide. A week after the last immunization, the whole serum of the mice were pooled and stored below –20°C until needed for use. The pooled serum were used in the subsequent experiments.

Assay for β -N-acetylhexosaminidase release. The activity of β -N-acetylhexosaminidase released from stimulated RBL-2H3 cells was determined by the method described by Tanaka *et al.*¹¹⁾ with appropriate modifications. In brief, from confluent cultures, 1×10^5 trypsinized RBL-2H3 cells dispersed in 80 μ l of the medium were inoculated into each well of a 96-well micro-plate. After 24 hours, 20 μ l of the medium containing the IgE antibody was added to each well. The micro-plate was then incubated at 37°C and washed three times with 100 μ l of HEPES-buffered saline (HBS) made up from a 10 mM HEPES buffer at pH 7.4 containing 135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, and 0.5 mM MgCl₂. 100 μ l of HBS with or without the antigen was added to almost all the wells, and HBS containing 0.2% Nonidet-P40 was added to the remaining wells to dissolve the cells for determining the total amount of the enzyme stored in them. After incubating at 37°C, 40 μ l of the conditioned medium or the cell lysate was added to the wells of another micro-plate that had been dispensed with 10 μ l of a sodium citrate buffer at pH 4.5 containing 4 mM *p*-nitrophenyl-N-acetyl- β -D-glucosaminide, a specific substrate for β -N-acetylhexosaminidase. After incubating for a given period at 37°C, the reaction was stopped by adding 125 μ l of a 0.5 M sodium carbonate buffer at pH 10.0. The enzyme activity was detected by measuring the absorbance at 415 nm with a CORONA MTP-120 microplate reader.

PCA test. The PCA test was carried out as previously described.¹⁾ In brief, Wistar rats were anesthetized by nembutal, and 50 μ l of the diluted

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Abbreviations: KSTI, Kunitz-type soybean trypsin inhibitor; PCA, passive cutaneous anaphylaxis; DNP, 2,4-dinitrophenol; HSA, human serum albumin; BSA, bovine serum albumin; FCS, fetal calf serum; MEM, minimum essential medium; EDTA, ethylenediamine-tetraacetic acid; PBS, phosphate buffered saline.

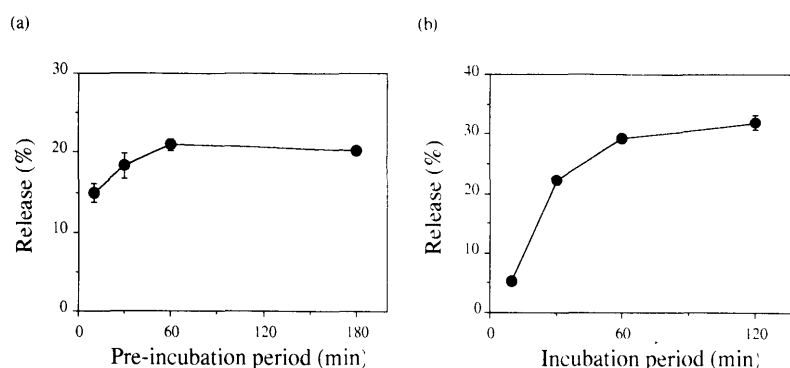


Fig. 1. Activation of RBL-2H3 Cells by the Combination of Anti-DNP Monoclonal IgE and DNP-conjugated HSA.

1×10^5 RBL-2H3 cells were pre-incubated with $1 \mu\text{g/ml}$ of anti-DNP monoclonal IgE at 37°C and subsequently incubated with $0.1 \mu\text{g/ml}$ of DNP-conjugated HSA at 37°C . The activity of β -N-acetylhexosaminidase released into the conditioned medium was measured. The pre-incubation period with the antibody was varied in (a) and the incubation period with the antigen was varied in (b). β -N-acetylhexosaminidase release is expressed as the percentage of the total activity of the enzyme contained in 1×10^5 cells. Values are presented as the mean $\pm$ SEM ($n=3$).

serum or of a solution containing monoclonal IgE was injected intracutaneously into the back skin. Three hours later, a saline solution including 0.1% of the antigen and 0.5% of Evans blue was intravenously injected. The blue spot wheels appearing on the back skin were measured to evaluate the allergic reaction.

Results and Discussion

We first optimized the assay conditions for evaluating the β -N-acetylhexosaminidase activity released from RBL-2H3 cells by using a 96-well micro-plate. As shown in Fig. 1a, priming of the cells with IgE, anti-DNA mAb, was completed within 60 min. Figure 1b shows that the integral activity of the enzyme released from the RBL-2H3 cells stimulated with the allergen reach a maximum within 60 min. Therefore, a 60-min incubation was adopted for both priming with the antibody and subsequent stimulation with the allergen for the assay of β -N-acetylhexosaminidase activity.

Figure 2 shows the results of PCA tests with both the anti-KSTI serum and anti-DNA mAb. Both tests were done on the same rat's skin to compare their titers under the same conditions. Neither antigen cross-reacted with the unpaired antiserum or antibody (anti-DNP mAb for KSTI and anti-KSTI serum for DNP-HSA; data not shown). The diameter of the spots generated by the allergic reaction became smaller in proportion to the dilution of the anti-KSTI serum. No allergic reaction was detected when the 1/100 diluted antiserum was injected. This is consistent with the results for anti-DNP mAb. The serum from non-immunized Balb/c mice did not react with either allergen.

The RBL-2H3 cell assay system was applied to investigate the reaginic activities of the anti-KSTI serum and anti-DNP mAb. The release of β -hexosaminidase decreased in response to the dilution of the anti-KSTI serum (Fig. 3a). A similar result was obtained in the case of anti-DNP mAb (Fig. 3b). There was, however, a significant difference between them in the magnitude of the cell response. By stimulating with the respective allergens, RBL-2H3 cells primed with the 1/10 diluted anti-KSTI serum exhibited only one fifth of the response observed with the cells primed with $0.1 \mu\text{g/ml}$ of anti-DNP mAb (Fig. 4). The results obtained by using the RBL-2H3 cell response must reflect merely the activation of the basophil caused by the cross-linking of Fc ϵ RI on its cell surface. Thus, with an

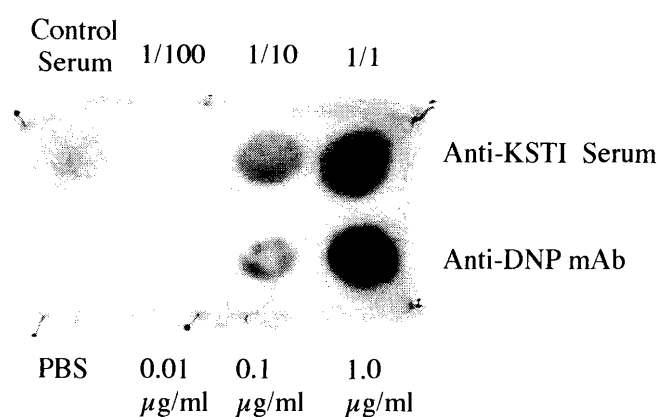


Fig. 2. Results of the PCA Test.

The mouse anti-KSTI serum at given dilutions and anti-DNP monoclonal IgE antibody at given concentrations were intracutaneously injected. An undiluted non-immunized control serum and vehicle phosphate-buffered saline (PBS) were also injected. After 3 hours, a solution containing 0.1% of the antigen and 0.5% of Evans blue was intravenously injected.

adequate amount of the allergen, $0.1 \mu\text{g/ml}$ of anti-DNP mAb would have evoked a stronger allergic reaction than the 1/10-diluted anti-KSTI serum. However, in the PCA test, both anti-KSTI serum and anti-DNA mAbs gave almost the same response. This was not due to any shortage of KSTI in the stimulation of RBL-2H3 cells, because the KSTI concentration applied could induce the maximal release of β -N-acetylhexosaminidase (Fig. 5). The lowest concentration exhibiting reaginic activity was the 1/50 dilution in the case of the anti-KSTI serum and $0.005 \mu\text{g/ml}$ in the case of anti-DNP mAb (data not shown). It was found that the RBL-2H3 cell response was as sensitive as the PCA test for the anti-KSTI serum, and was more sensitive than PCA for anti-DNP mAb. Taking into account the fact that antiserum obtained by immunizing with an antigen and aluminum hydroxide are known to contain a high level of antigen-specific IgG, IgG or an other factor excluding IgE might have affected the result of the PCA test by a pathway not mediated by basophils or mast cells.

It has been shown that RBL-2H3 cells could be used with an *in vitro* assay system reflecting an allergic reaction because they release such chemical mediators as histamine and serotonin in response to the cross-linking of Fc ϵ RI.⁶⁾ However, the histamine and serotonin assays involve some difficulties such as a complicated procedure or handling

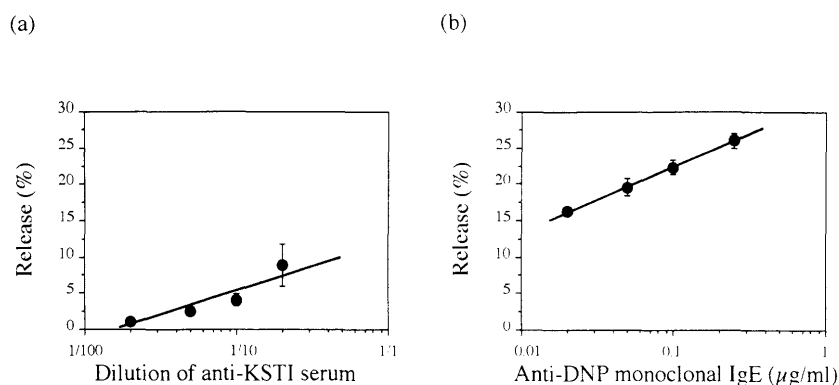


Fig. 3. β -Hexosaminidase Released from RBL-2H3 Cells after Treating with the Mouse Anti-KSTI Serum (a) and Anti-DNP IgE (b).

1×10^5 RBL-2H3 cells were pre-incubated with various concentrations of the mouse anti-KSTI serum (a), or anti-DNP IgE antibody (b), for 1 h at 37°C and subsequently incubated with 0.1 μ g/ml of each corresponding antigen for 1 h at 37°C. Values are presented as the mean $\pm$ SEM ($n=3$).

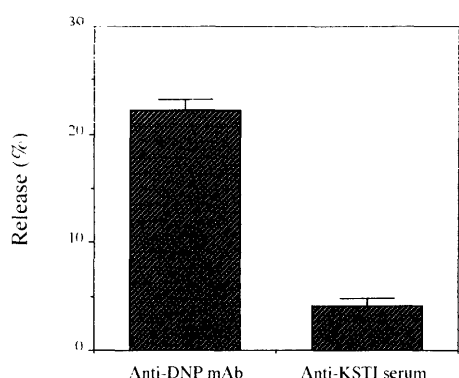


Fig. 4. β -N-Acetylhexosaminidase Release Evoked by Different Stimuli.

The released β -N-acetylhexosaminidase activity was compared between anti-DNP IgE (0.1 μ g/ml) and anti-KSTI serum (1/10 diluted), which gave similar results to the PCA reactions in Fig. 2. Bars show the mean $\pm$ SEM ($n=3$).

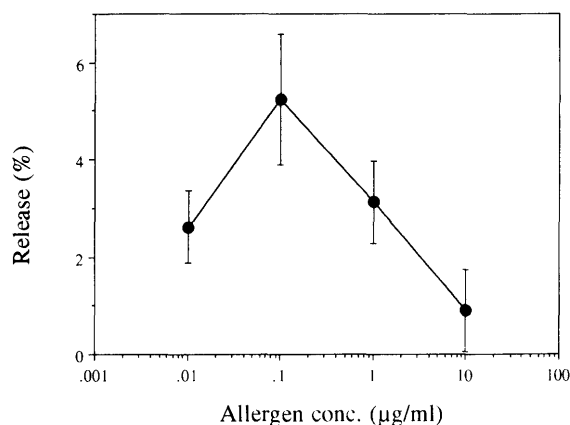


Fig. 5. Effect of KSTI on the Stimulation of RBL-2H3 Cells Primed by the Anti-KSTI Serum.

After priming with the 1/10-diluted anti-KSTI serum, 1×10^5 RBL-2H3 cells were incubated with various concentrations of KSTI as indicated in the figure. Values are presented as the mean $\pm$ SEM ($n=3$).

the radio-labelled compound.^{12–14}) The released activity of β -N-acetylhexosaminidase, which co-exists with histamine in the secretion granule of the mast cell and basophil,^{15,16} has recently been demonstrated to be useful as an index of cell response.^{11,14}) The assay of enzyme activity is much easier because it only requires measuring the absorbance of the product, *p*-nitrophenol, generated from the synthetic substrate, *p*-nitrophenyl-N-acetyl- β -D-glucosaminide.¹⁷) To yield reproducibility in PCA tests is very difficult due to

problems of the animal to animal variability and lack of skill in using the technique. Accordingly, the RBL-2H3 cell response can be substituted for the PCA test in scientific analysis because its detection limit is as good as that of the PCA test as shown in Fig. 3.

On the other hand, the presence of anti-KSTI reaginic activity in the serum of Balb/c mice immunized by KSTI could be sustained by measuring the response of RBL-2H3 cells primed by the antiserum. We thus confirmed the immunogenic and allergenic activity of KSTI, which is a representative proteinase inhibitor, toward Balb/c mice. We cannot yet explain the mechanism for IgE production induced by KSTI. Many proteins belonging to proteinase or proteinase-inhibitor families have been reported as allergens. For example, *Der p* I, a major allergen from *Dermatophagoides pteronyssinus*,¹⁸) is a thiol proteinase in the papain family, and ovomucoid (*Gal d* III), the most predominant allergen among egg-white proteins,^{19,20}) has trypsin inhibitory activity. The proteolytic activity or anti-proteolytic activity might play a role in protecting from the attack of the recipient's proteinase and might enable survival until receiving recognition from the immunosystem.

Experiments to study the allergenicity with model animals substituting for human beings as subjects have sometimes to be conducted. In the present study, we propose the optimal micro-assay conditions needing the least amount of sample serum to measure the β -N-acetylhexosaminidase activity released from RBL-2H3 cells. The improved *in vitro* measuring method utilizing RBL-2H3 cell response was found to be useful for analyzing the allergenicity of substances or the reaginic activity residing in a murine antiserum. The application of this method is expected to offer convenience for analyzing the epitope of various allergens, for studying the relationship between the protein structure and allergenicity, and for developing hypoallergenic products.

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