



An *In Vitro* Cell Based Model for Assessing the Potential of Allergens to Release Mediators Through the Cross-linking of IgE

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Abstract—The aim of this study was to examine the capacity of an *in vitro* model to test the potential of an allergen to cause cross-linking of IgE bound to the surface of mast cells. The model involved the passive sensitization of murine mast cells, with zearalenone IgE and subsequent exposure to anti IgE, zearalenone, zearalenone-BSA (with up to nine bound zearalenone molecules) or peanut lectin. The extent of cross-linking was determined by measuring the release of IgE mediators TNF α and histamine. Release of TNF α from IgE sensitized cells increased following exposure to zearalenone-BSA, but not following exposure to zearalenone alone or to peanut lectin. Histamine release could not be quantified against background. The results suggested that the model could be used to test allergenic potential through the availability of epitopes to bind and cross-link IgE on the surface of mast cells. As IgE is species specific, the model was adapted for use with a human cell system employing mast cells in lung fragments. TNF α release was measured, and the system was calibrated with the inhalant allergen from Timothy Grass and Timothy Grass specific IgE. © 1999 Published by Elsevier Science Ltd. All rights reserved

Keywords: food allergy; IgE; *in vitro* model; epitope; cross-linking.

Abbreviations: ATCC = American Tissue Culture Collection; BSA = bovine serum albumin; DMEM = Dulbecco's modified Eagle's medium; FBS = foetal bovine serum; IgE = immunoglobulin E; TNF α = tumour necrosis factor alpha.

INTRODUCTION

The methods currently used to assess the potential of foods to elicit an IgE allergic response include the double blind placebo controlled food challenge (DBPCFC), the skin prick test (*in vivo* techniques) and the radioallergosorbent assay (RAST), Western blotting and enzyme linked immunosorbent assay (ELISA) (*in vitro* techniques), (Bock, 1991; Nordlee *et al.*, 1996; Taylor and Lehrer, 1996). However, there are limitations to the application of these techniques. For example, *in vivo* methods involving human volunteers must be carried out in a clinical setting, which is costly in terms of recruitment, selection and facilities. Ethically, the use of DBPCFC to test foods is restricted since the subject may react severely (Metcalf *et al.*, 1996; Nordlee *et al.*, 1996). For these reasons, the *in vitro* methods

RAST, Western-blotting or ELISA have been used diagnostically and experimentally. However, RAST and ELISA cannot be used to examine mediator response via cross-linking of IgEs at the cell surface (the key stage in the IgE mediated immune response), and only a single epitope (IgE binding site) need be available for binding to give a positive result. Therefore, a method is needed to determine whether a protein can elicit a mediator response. This would eliminate false positives and enable modified proteins to be tested in a way that is not dependent on IgE binding alone. Alteration to proteins may be caused by genetic modification (where a protein is expressed against an unusual background), or by a novel preparation method (e.g. micro-particulation), which may expose, or change the spatial distribution of epitopes.

The *in vitro* model described is based on the IgE-dependent sensitization and activation of mast cells which results in the release the mediators TNF α and histamine (Okayama *et al.*, 1995a,b). To stimulate release of mediators in this model system, an

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allergen must possess more than one exposed IgE epitope in a spatial arrangement that permits binding to more than one IgE attached to a receptor on the surface of a mast cell.

MATERIALS AND METHODS

Murine mast cell IgE mediated release

The murine IgE used in the study was zearalenone specific monoclonal IgE (Sigma). Test compounds used were zearalenone (Sigma), zearalenone-BSA (Sigma), monoclonal rat anti-mouse IgE (Serotec) and peanut (*Arachis hypogea*) lectin (Biogenesis). Murine mast cells (MC/9) (ATCC) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM), without phenol red, supplemented with L-arginine-HCl (116 mg/litre); L-asparagine (36 mg/litre); folic acid (2 mg/litre); 1× non-essential amino acids (10 ml/litre), sodium pyruvate (1.0 mM); L-glutamine (2 mM); L-glucose (25 mM); HEPES (Sigma) (20 mM); 2-mercaptoethanol (0.05 mM); penicillin/streptomycin (100 U/100 µg/ml, respectively); 10% foetal bovine serum (FBS) (Sigma); and 5% Rat T-STIM™ with concanavalin A (Becton Dickinson). Unless otherwise stated, tissue culture products were obtained from Life Technologies. Cells were incubated at 37°C, 5% CO₂. For IgE mediated release cells (1×10^5 cells/well) were passively sensitized overnight using zearalenone specific monoclonal murine IgE, (titres used were 1:2000 and 1:100), and then washed twice with unsupplemented DMEM. Following this, cells were incubated for 4 hr with supplemented DMEM ± the test compounds (2 µg/ml and 4 µg/ml). Cell supernatants were obtained by centrifugation and stored at -20°C until assayed for IgE mediators. All exposures were carried out in duplicate with duplicate controls (non-sensitized cells ± test compounds and reagent blanks). Sensitized cells without test compounds acted as the negative control for the sensitized zearalenone-BSA exposed cells since free BSA (approx. 0.2–0.3 mg/well) was present. TNFα and histamine in the mast cell supernatants were determined by immunoassay kits (TNFα R&D Systems; histamine, Immuno-tech). Assays were carried out according to the manufacturer's instructions.

Human mast cell IgE mediated release

Freshly excised lung tissues providing a source of human mast cells were processed for experimentation within 2 hr of resection. The tissues were prepared for (a) the estimation of cell viability and mast cell content by enzymatic dispersal, and (b) micro-dissection to yield a minced tissue suitable for passive sensitization. Following the method of Ohkawara *et al.* (1992), tissues were washed three times in a 25 cm² tissue culture dish with DMEM containing 100 U penicillin/ml and 100 µg strepto-

mycin/ml. The lung was finely divided using sterile dissecting equipment and the fragments washed three times with DMEM containing 100 U penicillin/ml and 100 µg streptomycin/ml plus 2% FBS. These fragments were used either for passive sensitization or further dissociated as follows. Fragments were transferred to a 25-cm² cell culture flask and incubated for 75 min at 37°C in DMEM containing 100 U penicillin/ml and 100 µg streptomycin/ml, with collagenase 1 (63.6 µl 1.5 mg/ml/ml medium), hyaluronidase (3.75 µl 300 U/ml/ml medium) and FBS (2%). Dispersed cells were then separated from undissociated tissue by filtration through a wire mesh (40 gauge). Cells were washed twice in DMEM plus FBS (2%), collected by centrifugation (2800 rpm; 10 min; 20°C) and resuspended in DMEM plus FBS (2%). Viability was assessed by trypan blue exclusion and the proportion of mast cells assessed by positive staining with Kimura stain (Kimura *et al.*, 1973).

Passive sensitization of mast cells

Finely minced tissue samples were weighed and placed in 15-ml falcon tubes with 2 ml DMEM containing non-essential amino acids, glutamine 2 mM, 10% FBS plus 100 U penicillin/ml and 100 µg streptomycin/ml. IgE positive human serum was supplied by The Norfolk and Norwich Hospital Trust or purchased from DPC (USA), and 250 µl of a human serum containing a titred IgE was added to each tube (excluding controls). The tissue fragments plus IgE were incubated overnight at 37°C in a 5% CO₂ atmosphere.

Activation of mast cells

Excess IgE was removed from the lung fragments by washing twice with unsupplemented DMEM. Tissues were resuspended in 2 ml supplemented DMEM as previously described and either Timothy Grass allergen (DPC) or anti-human IgE (6.0 µg/ml) was added and the tissues incubated for 4 hr at 37°C, 5% CO₂. Following activation, the tissue was removed by centrifugation and the supernatant stored at -20°C until analysis. Each experiment was carried out with tissue from a single donor. TNFα levels in the supernatant were determined as before.

RESULTS

TNFα and histamine release from activated murine mast cells

The results are shown in Table 1. TNFα release was stimulated from zearalenone specific IgE sensitized mast cells exposed to zearalenone-BSA or anti-IgE. With an increase in antibody titre and test compound concentration, TNFα release was increased sevenfold and fivefold for zearalenone specific IgE sensitized mast cells exposed to zearalenone-BSA or anti-mouse IgE, respectively (Table 1).

Table 1. TNF α and histamine release following exposure of IgE sensitized murine mast cells to test compounds

Test compounds	IgE titre	TNF α release pg/ml	Histamine release μ M
Control	0	< LOD	2.1 $\pm$ 61%
Control	1:2000	< LOD	ND
Control	1:100	< LOD	0.8 $\pm$ 25%
Anti-IgE	0	< LOD	0.78 $\pm$ 32%
Anti-IgE	1:2000	9.5 $\pm$ 7.0%	ND
Anti-IgE	1:100	67.5 $\pm$ 16.0%	2.1 $\pm$ 19%
Peanut lectin	0	< LOD	1.6 $\pm$ 0%
Peanut lectin	1:2000	< LOD	ND
Peanut lectin	1:100	< LOD	< LOD
Zearalenone	0	< LOD	2.1 $\pm$ 61.0%
Zearalenone	1:2000	< LOD	ND
Zearalenone	1:100	< LOD	1.9 $\pm$ 36.0%
Zearalenone-BSA	0	< LOD	< LOD
Zearalenone-BSA	1:2000	47.5 $\pm$ 1.4%	ND
Zearalenone-BSA	1:100	242.0 $\pm$ 4.3%	1.5*

Values represent the mean $\pm$ SD (%) for paired replicates. *Single value. Limit of detection (LOD) for TNF α and histamine assays were 5.1 pg/ml and 0.2 nM, respectively. ND = not done.

The effect of zearalenone-BSA on TNF α release from zearalenone specific IgE sensitized mast cells was approximately four to five times greater than that of anti-IgE. When activation was attempted with peanut lectin or zearalenone alone, TNF α release was undetectable. Blanks also contained undetectable amounts of TNF α (data not shown). Histamine was spontaneously released from control mast cells both IgE sensitized without test compounds, and non-sensitized with test compounds. Zearalenone-BSA, anti-IgE, peanut lectin and zearalenone did not increase histamine release in this system.

TNF α release from activated human mast cells

Examination of five sets of dispersed lung tissues showed that the cells were always more than 70% viable. The lung tissues contained a variable proportion of mast cells but this was always in excess of 20% of the total cells present. The weight of tissue per replicate was optimized to obtain the

highest release of TNF α following sensitization and cross-linking with Anti-IgE. These results are presented in Fig. 1, and indicate that the optimum weight for the experimental conditions used is in the region of 0.2 g per replicate. The average error between replicates ($n = 3$) was 11.0%. Following optimization, an allergen concentration curve was constructed using purified Timothy Grass allergen (from DPC) following sensitization of the cells with two human sera (containing Timothy Grass specific IgE) obtained from separate sources (Norfolk and Norwich Hospital and DPC). Both sera had a RAST score of 3 (high allergic response), and contained between 3.5 and 17.5 kU/litre IgE. The results are presented in Fig. 2. Single point data for each of the sera indicate that a concentration of 100 μ l the Timothy Grass allergen/ml provided a maximal response for both sets of sensitized tissue, in terms of TNF α production; a higher concentration of Timothy Grass allergen was inhibitory.

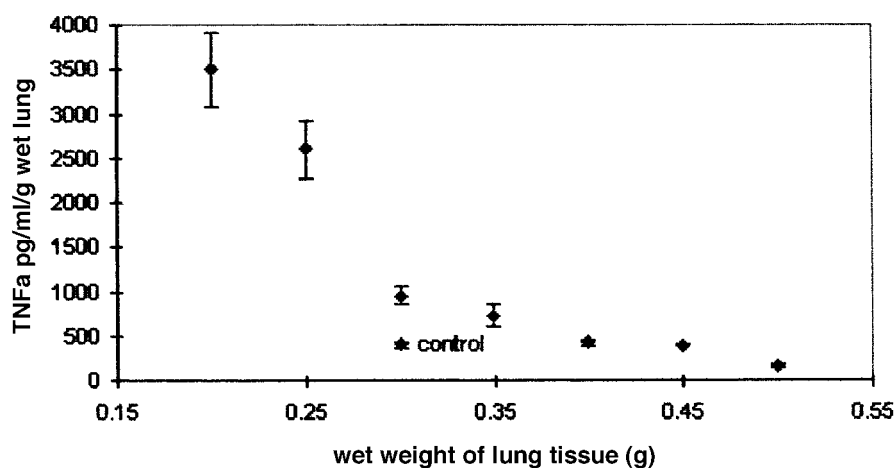


Fig. 1. The effect of tissue weight on IgE binding and TNF α release in the human *in vitro* assay system. Lung tissues were passively sensitized with an IgE-positive serum (RAST score 3), and cross-linked with anti-IgE. $N = 3$ for each point using tissue from one donor. The control was non-sensitized tissue incubated with anti-IgE only.

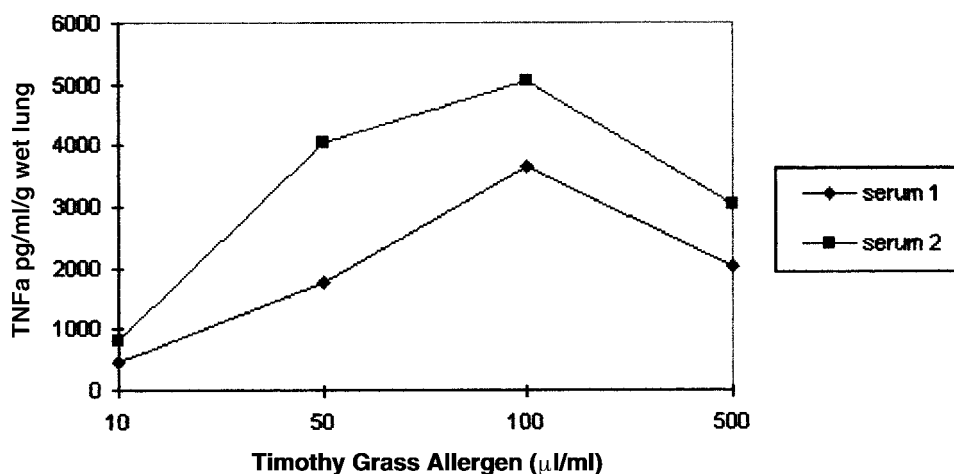


Fig. 2. The effect of Timothy Grass allergen on TNF α release from mast cells sensitized with Timothy Grass IgE. For each point 0.2 g of lung tissue from one donor was sensitized with two different Timothy Grass IgE-bearing sera at RAST score 3 (serum 1, obtained from DPC; serum 2, obtained from N&N Hospital). The sensitized tissues were incubated with increasing concentrations of Timothy Grass allergen and the cross-linking was measured in terms of TNF α production.

DISCUSSION

The aim of the present study was to set up a routine method for assessing the allergenicity of food proteins through the cross-linking of IgE bound to mast cells and the measurement of released mediators. Results indicate that the test system developed can be used to detect IgE specific antigens/allergens with TNF α as the indicator of an IgE response. The anti-zearalenone IgE sensitized cells did not respond to peanut lectin and zearalenone due to lack of epitope specificity and size, respectively (Ishizaka and Ishizaka, 1984). Only when conjugated to a protein bovine serum albumin (BSA) was zearalenone able to trigger an IgE response. The response of anti-zearalenone IgE sensitized mast cells to zearalenone-BSA was greater (3.5–5-fold) than that of anti-mouse IgE. This difference in response may reflect a higher specificity for zearalenone-BSA. Alternatively, it may have been due to a higher molar concentration of zearalenone (9 molecules of zearalenone per mole BSA) than anti-mouse IgE. The lack of an IgE-mediated response by zearalenone would not have been recognized using the standard *in vitro* techniques RAST, ELISA and Western blotting, since such techniques will detect binding of IgE to a single zearalenone molecule only, and not the potential of zearalenone to elicit an IgE-mediated response. This example highlights the need for further indication of potential allergenicity using a more complete method which is dependent on cross-linking and the production of mediators.

Unlike TNF α , histamine was spontaneously released by mast cells. It has been suggested that spontaneous histamine release by mast cells may be of biological significance specifically in the maintenance of histamine levels in body fluids (Kownatzki,

1982). The differences in histamine release between zearalenone-BSA and anti-mouse IgE activated IgE sensitized mast cells and their non-sensitized equivalents were not as great as the differences in TNF α release. Okayama *et al.* (1995a) showed that after 4 hr little or no histamine was released from IgE-activated human mast cells, but that during the next 48 hr histamine release increased significantly. In the present study murine mast cells were cross-linked for 4 hr, and a longer period may have resulted in a greater differential in histamine production. Peanut lectin did not stimulate histamine release from sensitized mast cells which may be due to lack of specificity or the inability of peanut lectin to bond non-specific sites of IgE. Shibasaki *et al.* (1992) examined 12 plant lectins and showed that some of these, but not peanut, could release histamine from basophils in an IgE dependent reaction which involves binding of the carbohydrate moieties of IgE rather than a specific recognition.

A key component of this model is that it tests the ability of an allergen to cross-link IgE bound to high-affinity receptors on the surface of a mast cell. If this does not occur then an inflammatory response will not be produced. To cross-link IgEs a presented allergen must possess at least two epitopes in a spatial arrangement that permits the binding of two or more IgEs. The model then represents a dynamic demonstration of the activity of an allergen in an *in vitro* system when assayed by TNF α release. The results show that zearalenone alone was insufficient to cross-link the IgEs on the surface of the mast cell; however, when zearalenone was conjugated to BSA (several molecules per BSA molecule) the ability to cause cross-linking was observed in terms of mediator release. This could be significant for novel presentations of familiar antigens, for example in genetically modified foods,

or for examining native, processed, digested, and purified forms of allergen for relative potency.

When an equivalent human cell-based model, using lung fragments as a source of mast cells, was investigated, it was clear that the production of TNF α could be used to optimize the weight of tissue used under standard conditions. The optimum weight (0.2 mg) permits a minimum of 12 replicates to be obtained from an average excision. This is sufficient to carry out a meaningful experiment including calibration points with each piece of lung tissue. The standard Timothy Grass allergen when used to challenge cells sensitized with the corresponding IgE-bearing sera can be used to calibrate the system for other allergens. The major Timothy Grass allergen belongs to the family of profilin proteins and cross-reactivity has been found between pollen profilins and a variety of fruit and vegetables including pear, celery, carrot and potato (Bush and Hefle, 1996; Heiss *et al.*, 1996), and is a well-characterized allergen in terms of RAST inhibition studies which determine the degree of binding to an IgE or family of IgEs. The calibration curve (Fig. 2) produced for Timothy Grass allergen suggests that there is an inhibition of mediator response above a particular threshold level (100 μ l/ml). One of the requirements of an allergen is to be multivalent, and thereby bind more than one IgE to bring about the cross-linking that results in mediator release. The addition of excess allergen to the *in vitro* system may result in reduced opportunity for allergen to bind to more than one IgE. This observation suggests that proteins produced in a novel form, and which may as a consequence have altered binding characteristics, might also exhibit a change in threshold inhibition against a standard protein that would be readily detected in the *in vitro* lung tissue system.

In conclusion, a mast cell-based approach can be used to mimic an IgE response measurable by TNF α release. The initial results are encouraging and characterization of the human system will be undertaken with the ultimate aim of developing a method that can be used to assess the allergenicity of food proteins, and the potential for change that may occur through novel processes such as genetic modification.

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REFERENCES

- Bock S. A. (1991) Oral challenge procedures. In *Food Allergy: Adverse Reactions to Foods and Food Additives*. Edited by D. D. Metcalfe, H. A. Sampson and R. Simon, pp. 81–95. Blackwell Scientific Publications, Boston.
- Bush R. K. and Hefle S. (1996) Food allergens. *Critical Reviews in Food Science and Nutrition* **36**, S119–S163.
- Heiss S., Fischer S., Muller W. D., Weber B., Hirschwehr R., Spitzauer S., Kraft D. and Valenta R. (1996) Identification of a 60kd cross-reactive allergen in pollen and plant-derived food. *Journal of Allergy and Clinical Immunology* **98**, 938–947.
- Ishizaka T. and Ishizaka K. (1984) Activation of mast cells for mediator release through IgE receptors. *Progress in Allergy* **34**, 188–235.
- Kimura I., Moritani Y. and Tanizaki Y. (1973) Basophils in bronchial asthma with reference to reagin-type allergy. *Clinical and Experimental Allergy* **3**, 195–202.
- Kownatzki E. (1982) Triggering of mast cells. *Molecular Immunology* **19**, 1297–1300.
- Metcalfe D. D., Astwood J. D., Townsend R., Sampson H. A., Taylor S. L. and Fuchs R. L. (1996) Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition* **36**, S165–S186.
- Nordlee J. A., Taylor S. L., Townsend J. A., Thomas L. A. and Bush R. K. (1996) Identification of a Brazil-nut allergen in transgenic soybeans. *New England Journal of Medicine* **334**, 688–692.
- Okayama Y., Petit-Frere C., Kassel O., Semper A., Quint D., Tunon-de-Lara M. J., Bradding P., Holgate S. T. and Church M. K. (1995a) IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells. *Journal of Immunology* **155**, 1796–1808.
- Okayama Y., Semper A., Holgate S. T. and Church M. K. (1995b) Multiple cytokine mRNA expression in human mast cells stimulated via Fc ϵ RI. *International Archives of Allergy and Immunology* **107**, 158–159.
- Ohkawara Y., Yamauchi K., Tanno Y., Tamura G., Ohtani H., Nagura H., Ohkuda K. and Takishima T. (1992) Human lung mast cells and pulmonary macrophages produce tumor necrosis factor α in sensitised lung tissue after IgE receptor triggering. *American Journal of Respiratory Cell and Molecular Biology* **7**, 385–392.
- Shibasaki M., Sumasaki R., Isoyama S. and Takita H. (1992) Interactions of lectins with human IgE; IgE binding property and histamine releasing activity of twelve plant lectins. *International Archives of Allergy and Immunology* **98**, 18–25.
- Taylor S. L. and Lehrer S. B. (1996) Principles and characteristics of food allergens. *Critical Reviews in Food Science and Nutrition* **36**, S91–S118.