

Rat basophil leukaemia (RBL) cells sensitized with low affinity IgE respond to high valency antigen

A. M. COLLINS, M. BASIL, K. NGUYEN and D. THELIAN

School of Microbiology and Immunology, University of New South Wales, Sydney, Australia

Summary

Background The very low concentrations of IgE antibodies in serum make investigations of the affinity of allergen-specific antibodies extremely difficult. In the absence of such studies, the fact that low IgE concentrations are capable of inducing powerful effector function has encouraged the view that IgE antibodies are typically high affinity antibodies. Yet the phenomenon of allergic cross-reactivity suggests that lower affinity IgE antibodies may sometimes be of clinical significance.

Objectives To investigate the effect of antibody affinity upon mast cell sensitivity in an *in vitro* model.

Methods Rat basophil leukaemia (RBL) cells were sensitized with one of three monoclonal IgE antibodies which bind to trinitrophenylated proteins with varying affinity. Serotonin release was measured after challenge of sensitized cells with trinitrophenylated human serum albumin (TNP-HSA).

Results Low valency TNP₃-HSA failed to stimulate degranulation of RBL cells sensitized with SPE-7 anti-DNP IgE, which binds TNP with low affinity. However, upon challenge with high concentrations (1250 ng/mL) of TNP₈-HSA, or as little as 10 ng/mL of highly substituted TNP₂₃-HSA, low levels of degranulation were seen. A similar relationship between antigen valency and cell sensitivity was seen with cells sensitized with the H-1 ϵ -DNP anti-DNP IgE, which binds with moderate affinity to TNP proteins.

Conclusion High valency antigen is capable of activating RBL cells sensitized with low affinity antibody. This has important implications for our understanding of allergic sensitization. It also suggests that the long-recognized relationship between antigen valency and RBL cell sensitivity may partly reflect the high functional affinity of cell-bound IgE when directed against multivalent antigen.

Keywords: affinity, allergy, avidity, epitope, IgE, mast cell, valency

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Introduction

It has been recognized for many years that affinity is a variable of fundamental consequence to antibody function [1]. The measurement of antibody affinity is therefore critical to a complete understanding of any humoral immune response. It remains difficult, however, to determine antibody affinity, particularly in the case of antibodies directed against complex multivalent antigens. This is

particularly true in the case of IgE antibodies [2,3] the concentrations of which remain relatively low even in allergic individuals [4].

The fact that such low IgE concentrations are capable of powerful effector function has encouraged the view that IgE antibodies are typically high affinity antibodies. This assumption is perhaps strengthened by the fact that both affinity maturation and class switching to IgE are T-cell dependent. Some evidence, however, indicates that clinically significant IgE antibodies may not always be of high affinity. This is particularly suggested by the phenomenon of allergic crossreactivity, for antibodies generated in

Correspondence: Dr A. M. Collins, School of Microbiology and Immunology, University of New South Wales, Sydney 2052, Australia.

response to one antigen should typically bind with lower affinity to a crossreactive antigen.

We have recently reported results of studies which examined the relationship between antigen valency and the sensitivity of Rat Basophil Leukaemia (RBL) cells to IgE-mediated degranulation [5]. In the course of these studies we hypothesized that the functional affinity of RBL cell-bound IgE must be dependent upon antigen valency. Surprisingly, despite the recognition that mast cell degranulation is dependent upon the cross-linking of cell-bound IgE by multivalent antigen, and despite the general acceptance that multivalent binding is essential to numerous receptor/ligand interactions, the relationship between antibody affinity, antigen valency and cell function does not appear to have been addressed in investigations of the mast cell. In fact, in studies of IgE-mediated degranulation of RBL cells, it has been stated that the observed relationship between cell sensitivity and the valency of challenge antigen is entirely the outcome of the ability of highly multivalent antigen to promote the formation of large $Fc\epsilon R$ aggregates [6].

Our observation that SPE-7 and H-1 ϵ -DNP monoclonal IgE anti-DNP antibodies bind with low affinity to trinitrophenylated proteins has allowed us to more fully examine the relationship between antibody affinity and mast cell degranulation using the RBL cell model. The results presented here reaffirm our view that antigen valency is of fundamental importance to mast cell sensitivity, and demonstrate that even relatively low affinity antibodies may be able to promote degranulation upon challenge with high valency antigen.

Materials and methods

Antibody and antigen preparations

TNP-HSA was prepared at varying levels of TNP substitution by the reaction of 2,4,6-trinitrobenzene sulphonic acid sodium salt (Sigma Chemical Co., St Louis, USA) with human serum albumin (HSA: Sigma), and was characterized spectrophotometrically using standard techniques [7]. Purified SPE-7 IgE anti-dinitrophenyl (DNP) antibodies were obtained from Sigma Chemical Co. Purified monoclonal LB4 murine anti-TNP IgE, H-1 ϵ -DNP murine anti-DNP IgE antibodies, and B1E3 rat monoclonal anti-mouse IgE were kindly provided by Dr Dan Conrad, Virginia Commonwealth University, Virginia, USA. IgE anti-OVA antibodies were raised as ascites using the OVA6B cell line, kindly provided by Mr G. Georgiou, Royal Children's Hospital, Melbourne. IgE antibody concentrations were quantified by ELISA assays and by RBL degranulation assays as previously described [5].

ELISA assays

An indirect ELISA assay was developed for the measurement of monoclonal TNP-specific IgE antibodies. Briefly, 96-well microtitre plates (Nunc A/S, Roskilde, Denmark) were coated with TNP₂₃-HSA (2.5 μ g/mL in 0.01 M carbonate buffer, pH 9.6) by overnight incubation at 4°C. Plates were then blocked with 1% skim milk/0.01 M carbonate buffer for 1 h at 37°C. Samples diluted in PBS/1% skim milk/0.05% Tween-20 were incubated for 1 h at 37°C. Plate-bound IgE was detected by the addition of B1E3 anti-mouse IgE (10 μ g/mL), and then peroxidase-labelled goat anti-rat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, USA). Each step involved incubation for 1 h at 37°C. Tetramethylbenzidine substrate (Sigma) was finally added to the wells for 10 min, after which the reaction was stopped using 2 M H₂SO₄. Optical densities were read at 450 nm using a Bio-Rad 3550 Microplate reader (Bio-Rad Laboratories, Richmond, CA, USA).

The affinity of H-1 ϵ -DNP, SPE-7 and LB4 binding for TNP was measured by an ELISA inhibition assay, as previously described [5]. The concentrations of the antibodies required for near-saturation binding to TNP₂₃-HSA-coated plates were first determined by the IgE anti-TNP ELISA assay. Aliquots of antibodies at these concentration were then incubated for 45 min at 37°C in the presence of varying concentrations (10⁻³ M to 10⁻⁹ M) of free monovalent TNP-lysine. Antibody/antigen preparations were then added to the ELISA plates and the assay completed as previously described. The affinity of the antibodies was expressed as the molar concentration (the 'I(50)_{TNP}') of TNP-lysine necessary for 50% inhibition of antibody-binding to the TNP-HSA-coated plates.

The relative affinities of H-1 ϵ -DNP and SPE-7 binding to TNP were also measured by a further modification of the ELISA assay, in which antigen-bound antibodies were eluted from the plate upon exposure to increasing concentrations of the chaotropic thiocyanate ion [8]. This required the introduction of an additional step to the standard ELISA assay, after the incubation of near-saturating concentrations of anti-TNP antibodies, and subsequent washing of the plates. Ammonium thiocyanate in 0.1 M phosphate buffer, pH 6.0, was then added to each well. The molarity of the chaotropic ions ranged from 0 to 6.5 M. The plates were incubated for 15 min at room temperature and then washed, after which the assay was completed as described for the standard ELISA. The affinity of the antibodies (the 'I(50)_{SCN}') was expressed as the molar concentration of thiocyanate ions producing a 50% reduction in absorbance when compared with thiocyanate-free wells.

Determination of serotonin release

RBL-2H3 cells were cultured in Dulbecco's Modified

Eagles Medium (DMEM) containing 10% Fetal Calf Serum (FCS: Cytosystems, Castle Hill, Australia) in 75 cm² culture flasks (Greiner GmbH, Frickenhausen, Germany). Cells were harvested, counted and resuspended in DMEM/FCS containing 2 μ Ci/mL ³H-5-hydroxytryptamine creatinine sulphate (Du Pont, Boston, MA, USA), then seeded into 96-well Nunclon microtitre plates (Nunc A/S, Roskilde, Denmark) at a concentration of 5×10^4 cells/well, and incubated overnight. ³H-serotonin-pulsed RBL cells were sensitized with saturating concentrations of monoclonal IgE antibodies (1 μ g/mL) and cultured for a further 3.5 h, before being challenged with TNP₃-HSA, TNP₈-HSA or TNP₂₃-HSA at concentrations ranging from 10 to 5000 ng/mL. After 30 min, 20 μ L supernatant samples were transferred to Wallac 1450 PET 96-well microtitre plates (Wallac Oy, Turku, Finland) containing 200 μ L Hisafe III scintillant (Wallac) per well. The RBL cells were then lysed with 1% Triton X-100 (Sigma) and 20 μ L lysate samples transferred to a second Wallac microtitre plate containing 200 μ L scintillant per well, for the measurement of unreleased ³H-serotonin. Plates were read in a Wallac Microbeta Plus microplate liquid scintillation counter. The percentage release of serotonin from challenged cells was then calculated. In additional experiments, B1E3 monoclonal anti-mouse IgE antibodies (100 ng/mL to 10 μ g/mL) were used to cross-link cell-bound IgE. Each plate included IgE-free and antigen-free wells as negative controls. Positive control wells containing IgE-sensitized cells were challenged with 100 ng/mL DNP₃₂-HSA, TNP₂₃-HSA or with 1 μ M calcium ionophore A23187 (Sigma). Fourfold replicates were set up for each antibody and antigen concentration, and for all controls.

As an alternative measure of cell sensitivity, RBL cells were sensitized with percentages of specific anti-DNP/TNP IgE ranging from 0.5% to 100%. This was achieved by the incubation of RBL cells in the presence of saturating concentrations (1 μ g/mL) of mixtures of anti-DNP/TNP antibodies and anti-OVA IgE as previously described [5].

Results were analysed using the unpaired *t*-test and statistical significance was accepted at the 5% level.

Results

The relative affinity of anti-DNP and anti-TNP IgE antibodies for the TNP-hapten

Near-saturating concentrations of LB4, H-1 ϵ -DNP and SPE-7 antibodies were determined by anti-TNP ELISA assay to require antibody concentrations of 100 ng/mL, 250 ng/mL and 2 μ g/mL, respectively (data not shown). Functional affinity was first measured by TNP-lysine inhibition assay, which showed the LB4 monoclonal to have an $I(50)_{\text{TNP}}$ of 3.0×10^{-5} M. The H-1 ϵ -DNP and SPE-7 antibodies were

shown to have substantially lower affinities, with $I(50)_{\text{TNP}}$ of 5.0×10^{-7} and 8.5×10^{-7} M, respectively. The closeness of these latter results, and the discrepancy between this result and the very high concentration of purified SPE-7 antibody required for saturation in the anti-TNP ELISA, led us to undertake a second series of measurements to clarify the functional affinity for the two low affinity antibodies, using the thiocyanate elution technique. The results of the ELISA chaotropic elution assay are presented as Fig. 1. The H-1 ϵ -DNP IgE was shown to have an $I(50)_{\text{SCN}}$ of 1.7 M, while the SPE-7 IgE had an $I(50)_{\text{SCN}}$ of 0.3 M. We believe the thiocyanate elution assay, in which chaotropic ions disrupt hydrophobic bonds between antigen and antibody [9], is an inappropriate technique for the determination of the functional affinity of the LB4 antibody, for this antibody has been shown to have an unusually high number of aromatic residues at its binding site [10], and hence will be particularly susceptible to the effects of chaotropic ions. This view was confirmed when it was shown that very low concentrations of thiocyanate were sufficient to disrupt the binding of this antibody to the solid phase (results not shown). Taken together, these results show LB4 to have high affinity, H-1 ϵ -DNP IgE to have moderate affinity, and SPE-7 to have low affinity for the TNP hapten.

RBL cell degranulation

No differences were seen between the three anti-DNP/TNP antibodies when B1E3 rat monoclonal anti-mouse IgE antibodies, rather than antigen preparations, were used as the cross-linking agent (data not shown). Cells sensitized with either LB4 anti-TNP IgE, H-1 ϵ -DNP or SPE-7

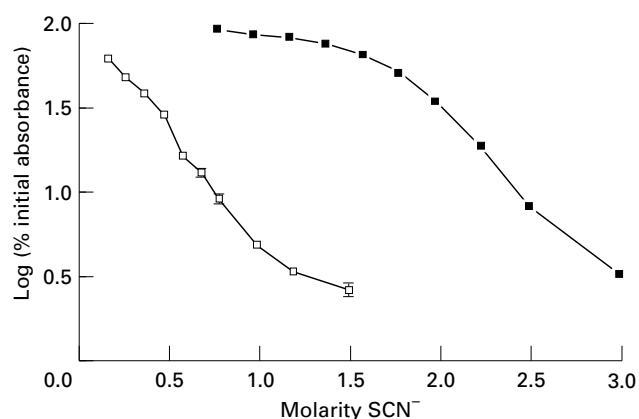


Fig. 1. Elution of TNP₂₃-HSA-bound H-1 ϵ -DNP and SPE-7 antibodies in the presence of concentrations of chaotropic SCN⁻ ranging from 0.1 M to 3 M. Data shown are means of fourfold replicates. Error bars in this and all subsequent figures are not indicated when graphically too small. □—SPE-7. ■—H-1 ϵ .

anti-DNP IgE were challenged with TNP₃-HSA at concentrations ranging from 10 to 5000 ng/mL. The results are shown as Fig. 2a. Even at the highest antigen concentration, no degranulation was seen from SPE-7-sensitized cells; 15.9% degranulation of cells sensitized with H-1 ϵ -DNP IgE was seen upon exposure to 1250 ng/mL TNP₃-HSA, which was significantly above background level (9.5%). At the highest concentration of challenge antigen (5000 ng/mL), 22.7% degranulation was seen. Cells sensitized with LB4 anti-TNP IgE showed degranulation above background levels when challenged with just 40 ng/mL TNP₃-HSA (13.6% degranulation), while at a challenge concentration of 5000 ng/mL, 58.4% degranulation was measured. Significant degranulation was seen from cells sensitized with as little as 5% LB4 anti-TNP IgE and 25% H-1 ϵ -DNP anti-DNP IgE when challenged with 5000 ng/mL TNP₃-HSA (Fig. 2b). Percent

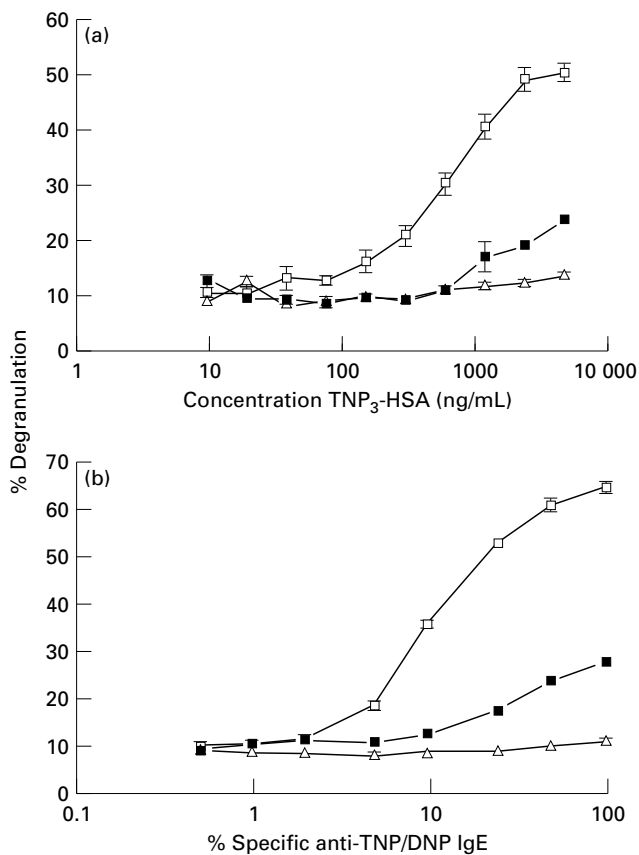


Fig. 2. ³H-serotonin release from RBL cells sensitized with (a) 1 µg/mL monoclonal IgE antibody and challenged with concentrations of TNP₃-HSA varying from 10 ng/mL to 5000 ng/mL; and with (b) percentages of antigen-specific monoclonal IgE varying from 0.5% to 100%, and challenged with 5000 ng/mL TNP₃-HSA. Data shown are means of four-fold replicates. —□—, LB4. —■—, H-1 ϵ . —△—, SPE-7.

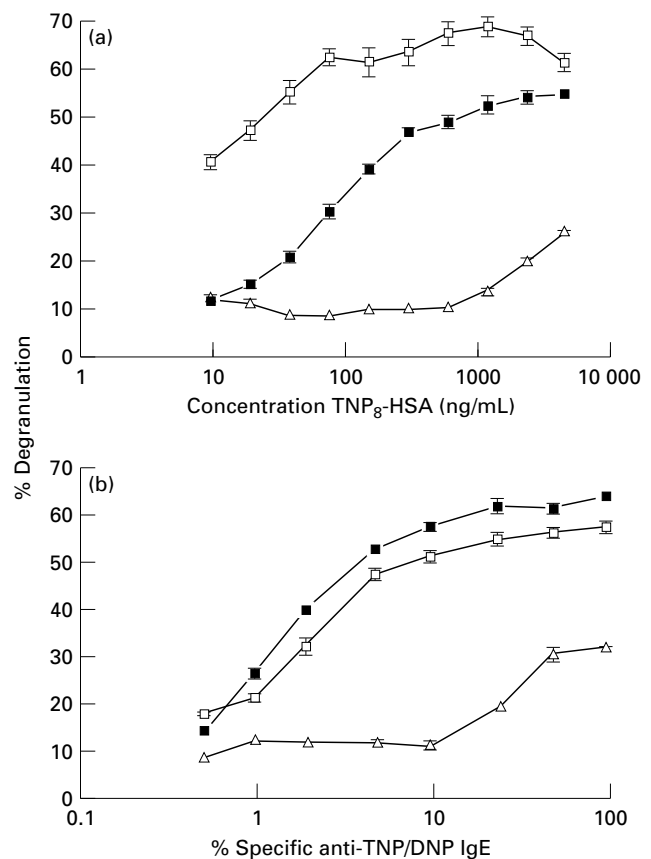


Fig. 3. ³H-serotonin release from RBL cells sensitized with (a) 1 µg/mL monoclonal IgE antibody and challenged with concentrations of TNP₈-HSA varying from 10 ng/mL to 5000 ng/mL; and with (b) percentages of antigen-specific monoclonal IgE varying from 0.5% to 100%, and challenged with 5000 ng/mL TNP₈-HSA. Data shown are means of fourfold replicates. —□—, LB4. —■—, H-1 ϵ . —△—, SPE-7.

release was 18.5% and 17.1%, respectively, while background degranulation was 9.5%.

Low levels of degranulation were seen from SPE-7-sensitized cells challenged with high concentrations of TNP₈-HSA (Fig. 3a), and when sensitized with 25% or more specific IgE (Fig. 3b). Serotonin release from cells sensitized with 100% LB4 IgE was significantly higher than from H-1 ϵ -DNP sensitized-cells, except at the highest concentration of challenge antigen. Maximum degranulation of LB4-sensitized cells was seen upon exposure to 1250 ng/mL TNP₈-HSA (Fig. 3a). At 5000 ng/mL challenge concentration, H-1 ϵ -DNP IgE-sensitized cells degranulation was slightly but significantly higher than LB4-sensitized cells when armed with between 1% and 100% TNP/DNP-specific antibody (Fig. 3b).

Despite the low affinity of SPE-7 IgE for TNP, challenge with as little as 80 ng/mL TNP₂₃-HSA led to maximum

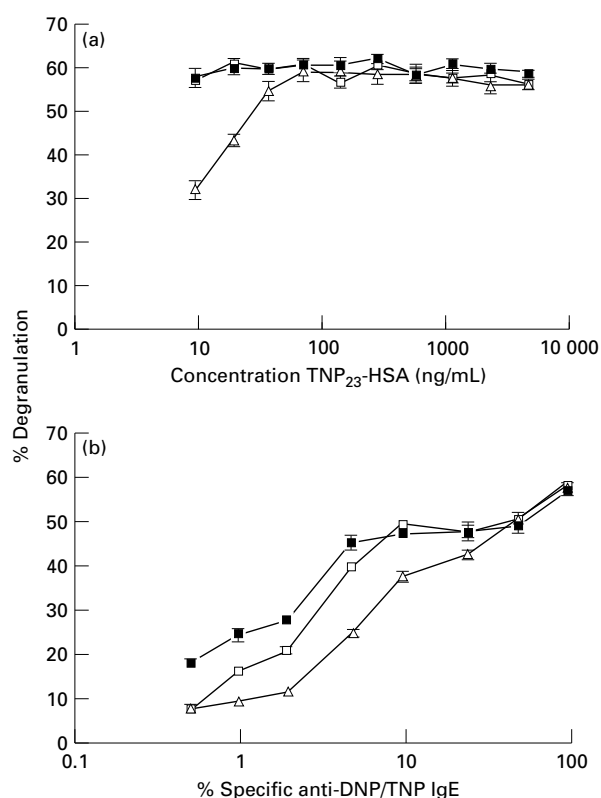


Fig. 4. ³H-serotonin release from RBL cells sensitized with (a) 1 μ g/mL monoclonal IgE antibody and challenged with concentrations of TNP₂₃-HSA varying from 10 ng/mL to 5000 ng/mL; and with (b) percentages of antigen-specific monoclonal IgE varying from 0.5% to 100%, and challenged with 5000 ng/mL TNP₂₃-HSA. Data shown are means of fourfold replicates. —□—, LB4. —■—, H-1 ϵ . —△—, SPE-7.

release of serotonin (Fig. 4a). Cells sensitized with just 5% SPE-7 released almost 25% serotonin upon exposure to 5000 ng/mL TNP₂₃-HSA (Fig. 4b). Significant differences were only seen between H-1 ϵ -DNP-sensitized cells and LB4-sensitized cells when less than 5% cell-bound IgE was antigen specific (Fig. 4b). Higher levels of degranulation were seen from H-1 ϵ -DNP-sensitized cells, despite their lower affinity for the TNP hapten.

Discussion

In this study we have investigated the degranulation of cells sensitized with low, moderate and high affinity antibodies for the TNP hapten. We have shown that cells sensitized with low affinity antibodies, which are unable to respond to challenge with low valency antigen, degranulate in the presence of higher valency antigen. In fact when challenged with high valency antigen, such cells behave little differently to cells sensitized with high affinity antibodies. We

believe these results can best be understood to result from co-operative interactions between antibodies directed against different epitopes on a multivalent antigen. Just as IgM attains a relatively high avidity for multivalent antigen by self-aggregation [11], IgE antibodies may acquire a high functional affinity by their association on the surface of mast cells.

Our results suggest that if relatively low affinity antibodies on the surface of a mast cell are directed against sufficient determinants on an allergen, they may together function as high avidity receptors for that allergen. This in turn suggests that previous explanations for the relationship between IgE-mediated degranulation and antigen valency are incomplete. This relationship has been repeatedly claimed to reflect a correlation between activation signal strength and Fc ϵ R aggregate size, which should be increased in the presence of high valency antigen [6, 12].

The possibility that antigen valency, antibody affinity and cell sensitivity interact in this way has important implications for our understanding of allergic sensitization in general, and of the phenomenon of allergic crossreactivity in particular. Allergic crossreactivity is perhaps most widely known in the context of drug hypersensitivity, where for example, many penicillin-allergic patients are also allergic to cephalosporins [13] and other betalactam antibiotics [14]. In light of the relationship between antigen valency and the sensitivity of IgE-armed cells, it is of interest that drug hypersensitivity seems typically to require the formation of highly substituted drug complexes with self-carrier proteins [15].

Allergic crossreactivity is not confined to reactivity against low molecular weight allergenic haptens. It is well recognized, for example, that some patients who are allergic to tree pollen may also be sensitive to tree nuts [16]. Latex sensitivity may be accompanied by sensitivity to disparate fruits [17], while those allergic to the house dust mite may react to other mites species [18] and even to phylogenetically more distantly related scabies species [19].

It has been suggested that some IgE antibodies uniquely display an ability to bind to numerous unrelated chemical structures, and that this may explain the phenomenon of allergic crossreactivity [20]. More commonly, crossreactivity has been understood to be a consequence of homology between antigenic determinants carried on chemically and perhaps phylogenetically related proteins. For a number of reasons, however, it is difficult to see how the fact of immunochemical crossreactivity can be a sufficient explanation for the phenomenon of allergic crossreactivity. Antibodies generated in response to one antigen should typically bind with lower affinity to homologous antigens. Since mast cells may be sensitized with antibodies directed against a multitude of antigens, it has been difficult to understand how the low percentage of specific IgE directed against epitopes

of one antigen could mediate degranulation of mast cells if their affinity for a cross-reactive antigen was low. This difficulty has been compounded by the fact that allergic sensitization implies an ability of the sensitized mast cell to respond to extremely low concentrations of allergen [21], and to respond with substantial degranulation [22].

The results of this study suggest that an IgE response directed against an unusually high number of antigen epitopes could promote an enhancement of the functional affinity of cell-bound IgE, and so promote the phenomenon of allergic crossreactivity. Such a response would more generally account for the exquisite sensitivity of mast cells for specific antigen in allergic individuals.

'Valency' is a characteristic conventionally applied to both antigens and antibodies. Antibody valency is a description of a physicochemical fact that each antibody isotype carries an invariant number of antigen binding sites. This is not the case with antigen valency. The set of B-cell epitopes on any antigen varies from species to species [18,23], and from strain to strain or individual to individual [24,25]. The exact nature of B-cell epitopes, in fact, remains an enigma, despite the substantial progress which has recently been made in our understanding of T-cell epitopes [26].

The identification of B-cell epitopes, and particularly of conformational epitopes remains a technically difficult process. Sequential epitopes have been studied using recombinant peptides, and it has been shown that allergic individuals collectively respond to numerous sequential epitopes [27,28]. The number of sequential determinants against which an individual responds, however, does not appear to have been reported.

Investigations of conformational epitopes require indirect procedures, such as comparisons of the binding of antibodies to native and denatured antigen [29]. The identification of conformational epitopes is best achieved using monoclonal antibodies to inhibit the binding of serum antibodies to antigen. The principle impediment to the application of this technique is the lack of suitable epitope-defining monoclonal antibodies. Even in the case of the most clinically important allergens, only a handful of such monoclonal antibodies have been developed [30]. The enumeration of the valency of a human response, which necessitates the identification of all B-cell epitopes, therefore remains a task for the future.

Only with the development of techniques by which the entire surface of an antigen can be mapped will it be possible to properly investigate the fine specificity of the allergic response. Ultimately it will be important for such investigations to include the study of each antibody class and subclass, for it has recently been shown that Fc γ R on mast cells are able to inhibit Fc ϵ R-mediated degranulation [31]. A thorough understanding of the fine specificity of the response of allergic and non-allergic individuals to

environmental allergens will also enable us to learn whether the relationship between valency and cell sensitivity, which is so striking in the *in vitro* studies reported here, is equally of significance *in vivo*.

Acknowledgements

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