A. Buku
B.A. Condie
J.A. Price
M. Mezei

[Ala[12]]MCD peptide: a lead peptide to inhibitors of immunoglobulin E binding to mast cell receptors[1]

Authors' affiliations:
A. Buku and M. Mezei, Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY, 10029 USA

B. A. Condie, The Wistar Institute, Philadelphia, PA, 19104 USA

J. A. Price, Department of Pathology, College of Osteopathic Medicine, University of Oklahoma, Tulsa, OK, 74107 USA

Correspondence to:
A. Buku
Department of Physiology and Biophysics
Mount Sinai School of Medicine
One Gustave L. Levy Place
Box 1218
New York
NY 10029
USA
Tel.: +1-212-241-5891
Fax: +1-212-860-3369
E-mail: angeliki.buku@mssm.edu

Dates:
Received 24 March 2005
Revised 9 May 2005
Accepted 29 May 2005

[1]A preliminary report of this study was presented in the 28th European Peptide Symposium, 2004, Prague, Czech Republic.

To cite this article:
Buku, A., Condie, B.A., Price, J.A. & Mezei, M. [Ala[12]]MCD peptide: a lead peptide to inhibitors of immunoglobulin E binding to mast cell receptors[1]. *J. Peptide Res.*, 2005, **66**, 132–137.
DOI 10.1111/j.1399-3011.2005.00281.x

Key words: β-hexosaminidase; FcεRIα receptor; fluorescence polarization; immunoglobulin E; mast cell degranulating peptide; rat basophilic leukemia cells

Abstract: An effort was made to discover mast cell degranulating (MCD) peptide analogs that bind with high affinity to mast cell receptors without triggering secretion of histamine or other mediators of the allergic reaction initiated by immunoglobulin E (IgE) after mast cell activation. Such compounds could serve as inhibitors of IgE binding to mast cell receptors. An alanine scan of MCD peptide reported previously showed that the analog [Ala[12]]MCD was 120-fold less potent in histamine-releasing activity and fivefold more potent in binding affinity to mast cell receptors than the parent MCD peptide. Because this analog showed marginal intrinsic activity and good binding affinity it was subsequently tested in the present study as an IgE inhibitor. In contrast to MCD peptide, [Ala[12]]MCD showed a 50% inhibition of IgE binding to the FcεRIα mast cell receptor by using rat basophilic leukemia (RBL-2H3) mast cells and fluorescence polarization. Furthermore, in a β-hexosaminidase secretory assay, the peptide also showed a 50% inhibition of the secretion of this enzyme caused by IgE. An attempt was made to relate structural changes and biologic differences between the [Ala[12]]MCD analog and the parent MCD peptide. The present results show that [Ala[12]]MCD may provide a base for designing agents to prevent IgE/FcεRIα interactions and, consequently, allergic conditions.

Abbreviations: Acm, acetamidomethyl; anti-DNP-IgE, mouse immunoglobulin E; Boc, *t*-butyloxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; DNP-HAS, dinitrophenyl human serum albumin; FcεRIα, high affinity receptor for IgE; fluoIgE, fluorescent IgE; HBSS, HEPES buffer salt solution; MEM, minimum essential medium; *p*-MeBzl, *p*-methylbenzyl; RBL-2H3, rat basophilic leukemia cells.

Binding of immunoglobulin E (IgE) to mast cells via high affinity FcεRI surface mast cell receptors is central to allergic disease. When IgE molecules bound to the surface of mast cells are cross-linked by multivalent allergens, the receptors aggregate. This aggregation initiates cellular activation leading to the release of granule-stored histamine and other inflammatory mediators, which are associated with type I hypersensitivity, i.e. allergic reaction (1). The main strategy for designing agents against allergic diseases is inhibiting the binding of IgE to mast cells. In the treatment of certain allergies, progress was made on the molecular level by using monoclonal antibodies to bind IgE in the circulation and prevent its binding to these cells (2). However, the broad spectrum of allergies and side effects caused by most antiallergic drugs are factors to be considered. Therefore, small molecules such as peptides that target the FcεRI receptor and block IgE-binding or IgE cross-linking offer an alternative treatment strategy. Over the years, efforts have been made by using peptides from different sources as inhibitors of the IgE/FcεRI interaction. These studies indicated at last that disulfide bonded peptides showed higher inhibition than their linear counterparts (3–6). On the contrary, mast cell degranulating (MCD) peptide, a component of bee venom, is a suitable natural choice for studying allergy, because of its chemical and biologic properties. MCD peptide is a 22 amino acid bicyclic peptide containing two disulfide bridges between $Cys^{3,15}$ and $Cys^{5,19}$. It stimulates mast cell degranulation at low concentration (7) and at high concentration also inhibits histamine release in the presence of IgE (8). MCD peptide binds to mast cell receptors in a dose–response manner and has been found to some extent to inhibit binding of IgE to this receptor (9). However, because of the high binding affinity of IgE to the mast cell receptor ($K_d \sim 10^{-10}$ M) and consequent histamine release the inhibition of IgE requires compounds with high binding affinity to this receptor but without histamine- or other mediator-releasing activity.

To this end extended structure–activity studies with MCD peptide were undertaken (10–13) including a partial alanine scan by substituting the basic amino acids and the proline residue in the MCD peptide sequence (14,15). Of all MCD peptide alanine analogs synthesized, only the [Ala[12]]MCD analog with proline substitution in position 12 of the MCD peptide sequence showed the required biologic properties. In the present study, this peptide was tested as an inhibitor of IgE secretory activity and binding.

Experimental Procedures

[Ala[12]]MCD peptide synthesis and characterization

The [Ala[12]]MCD analog with the following primary structure: Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Ala[12]-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-$AsnNH_2$ was synthesized by stepwise solid-phase synthesis using *t*-butyloxycarbonyl/benzyl (Boc/Bzl) methodology. The $Cys^{3,15}$ residues were protected with the *p*-methylbenzyl (*p*-MeBzl) group and the $Cys^{5,19}$ with the acetamidomethyl (Acm) group. Purification, and analysis of the [Ala[12]]MCD peptide followed a well-established protocol used for the solid-phase synthesis of MCD peptide analogs and published previously (14).

RBL-2H3 cells and reagents

The rat basophilic leukemia (RBL-2H3) cell line (16) was a generous gift of Dr D. Holowka, Cornell University, Ithaca, NY, USA. The cells were grown on tissue culture plates in minimum essential medium (MEM) with Earle's salts medium supplemented with 2% (v/v) Gln, 0.1% (v/v) gentamicin (all from Gibco BRL, Carlsbad, CA, USA), and 20% (v/v) fetal bovine serum (Atlas BL, Fort Collins, CO, USA) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The media were changed three times per week until the cells reached 90% confluence. The adherent cells were washed with fresh medium and detached from the plates by gentle pipetting or scraping. They were used for experiments or subculturing.

β-Hexosaminidase release assay

Degranulation of the RBL-2H3 cells was measured by release of the enzyme β-hexosaminidase (17). For each experiment, aliquots of 1 mL cell suspension in MEM containing 10^5 cells were dispensed in 24-well plates containing various peptide concentrations or 0.460 μg/mL monoanti-dinitrophenyl (DNP)-mouse IgE (Sigma, St Louis, MO, USA) and incubated over night at 37 °C. During this period of time the cells were adhered to the bottom of the wells. The medium in wells containing peptide were aspirated and the cells sensitized with 0.460 μg/mL anti-DNP-IgE. After incubation for 90 min at 37 °C, all wells were washed twice with warm HBSS [20 mM HEPES, 135 mM NaCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM KCl, 5.6 mM glucose, and 0.1% bovine serum albumin (BSA), pH 7.4] by aspiration. The wells containing peptide/IgE mixtures or only IgE were challenged with

120 μL DNP-HSA (Sigma; 1 μg/mL in HBSS) for 30 min at 37 °C. Total cellular enzyme release was obtained by adding 1% Triton X-100 (w/v) in HBSS to wells with equal number of cells. Controls were run only with the same number of cells in HBSS to measure the spontaneous release by unstimulated cells. About 30 μL aliquots of the cell supernatants were removed from all wells in triplicate to 96-well plates for β-hexosaminidase release. The aliquots were incubated with 50 μL of the enzyme substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidase (Sigma; 1.3 mg/mL in phosphate/citrate buffer, pH 4.5) for 1 h at 37 °C. After quenching by addition of 80 μL 0.5 M sodium hydroxide, the absorbance of the released *p*-nitrophenol was read in a plate reader (Molecular Devices, Menlo Park, CA, USA) at 405 nm. Percentage enzyme release was calculated as the ratio of secreted minus spontaneous release (<6%) to total release of lysed cells × 100. The maximum release of hexosaminidase from cells treated only with anti-DNP-IgE after subtraction of spontaneous release was taken as 100%. The optical density (OD) data were analyzed using the ORIGIN software.

Peptide inhibition of IgE binding to human FcεRIα mast cell receptor

Increasing concentrations of [Ala[12]]MCD or MCD peptide and 7 nM FcεRIα soluble receptor protein (Heska Corporation, Fort Collins, CO, USA) in phosphate-buffered saline, pH 7.4, were incubated for 20 min at room temperature in borosilicate tubes (6 × 50 mm). After 4 nM fluorescent IgE (fluoIgE; Accurate Chemicals, Westbury, NY, USA) was added to a final volume of 100 μL, the fluorescence polarization was measured in a Beacon 2000 fluorescence polarization analyzer (PanVera, Madison, WI, USA). The filters used were 485 nm for excitation and 535 nm for emission with 3 nm bandwidth (14). The millipolarization units were the average of many consecutive readings of each sample. Following the same procedure, a comparative experiment was done by titrating 4 nM fluoIgE and 7 nM FcεRIα receptor protein with increasing concentrations of unlabeled IgE. The ID_{50}, which is the concentration of compounds required to inhibit 50% of receptor-bound fluoIgE was determined by nonlinear regression with the aid of ORIGIN software.

Computational protocol

The effect of the Pro[12] to Ala[12] mutation on the conformation of the peptide was examined using Monte Carlo simulations. Four different initial configurations were generated as follows. The INSIGHT II (Accelrys, San Diego, CA, USA) molecular modeling system was used to generate a straight helix, followed by manual rotation of torsion angles to bring the loop-forming cysteines close and thus to allow the formation of the disulfide bonds. This structure was first minimized by CHARMM (18) to ensure that all bond lengths and angles were closed to their canonical values. Next, the loop containing the Pro[12] residue was opened. The resulting structure was simulated at high temperature using the torsion angle Monte Carlo program MMC (19) with the solvent represented as a dielectric of $\epsilon = 80$ in order to scramble the system. Four different structures were saved, and each was again manually closed, followed by CHARMM minimization. The four structures were then each solvated in a periodic box, and equilibrated. The water molecules beyond two hydration shells of the equilibrated structures were filtered out and the remaining structures underwent long Monte Carlo simulations using the primary hydration shell technique (PHS) (20). The backbone torsion angles were sampled using local moves (21) enhanced by the reverse proximity criterion (22). The side chain torsions were sampled with the extension-biased scheme (23). The effect of mutation was studied by replacing the prolines in all four structures by alanine. This was achieved by CHARMM's ICBUILD option, followed again by a short minimization. The mutated structures were then treated by the same protocol as the wild type and the shifts in conformation were examined in all four structures.

Results and Discussion

The [Ala[12]]MCD peptide showed almost no intrinsic activity when histamine release was previously tested in rat peritoneal mast cells, but showed better binding affinity to the FcεRIα subunit of the IgE receptor than the parent MCD peptide determined by fluorescence polarization (14). Therefore, this analog was further tested as an inhibitor of the mast cell degranulation and of the binding of IgE to the mast cell receptor.

For degranulation experiments we used RBL-2H3 cultured cells. These cells are homologous to mucosal mast cells and also carry high affinity FεRI mast cell receptors (24). A measure for mast cell degranulation besides histamine release is the release of the β-hexosaminidase enzyme from mast cell granules. RBL-2H3 cells sensitized with IgE respond to DNP-HSA antigen-induced cross-linking and subsequent cell activation by releasing this

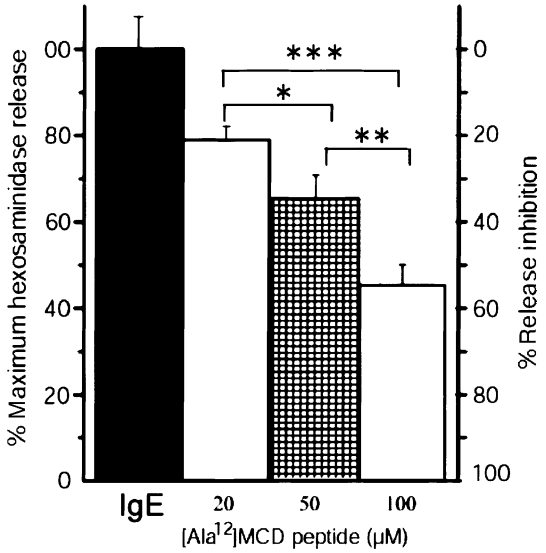

Figure 1. Inhibition of immunoglobulin E (IgE)-induced β-hexosaminidase release from rat basophilic leukemia (RBL-2H3) cells by [Ala[12]]-MCD. Methods are described under Experimental Procedures. Data are the mean ± SEM of many separate experiments. All assays were done with the same batch of cells at the same time. Secreted enzyme was expressed as a percentage of the total cellular enzyme content. The maximum release of hexosaminidase by IgE in the absence of peptide was taken as 100% release, i.e. 0% inhibition. Denotations by *, **, *** represent $P < 0.06$, $P < 0.02$, and $P < 0.0001$, respectively.

enzyme. We evaluated the concentration-dependent inhibition of IgE-induced enzyme release by using various concentrations of this peptide between 0 and 100 μM. The enzyme release was expressed either as a percentage of maximum release or of maximum release inhibition (Fig. 1). An attenuation of allergen-induced degranulation was started around 15 μM peptide. The maximum inhibition of enzyme release caused by IgE with the [Ala[12]]-MCD analog was about 50% at the highest concentration used (100 μM). In contrast, MCD peptide released this enzyme when tested at the same concentrations as [Ala[12]]MCD (data not shown). In the degranulation assay the RBL-2H3 cells were pre-incubated with the peptides before the cells were sensitized with IgE. Otherwise no inhibitory effect of antigen-induced cell activation was observed.

For binding inhibition experiments the FcεRIα-binding subunit of the human FcεRI mast cell receptor was used because this subunit was found to be sufficient for the binding of IgE to that receptor (25). The FcεRIα soluble protein made it possible to perform nonradioactive competitive-binding studies in a homogeneous solution with fluorescent ligands via fluorescence polarization (26). The ID_{50} for fluoIgE-binding inhibition by [Ala[12]]MCD and MCD peptides was 6 ± 1.0 μM and 132 ± 17 μM, respectively. There is a 20-fold increase in the inhibition of [Ala[12]]MCD compared with MCD peptide. In comparison, the ID_{50} of the IgE inhibition for fluoIgE was 3 ± 1.1 μM whereas the ID_{50} of [Ala[12]]MCD peptide was only twofold higher than that of IgE. These results suggest that the [Ala[12]]MCD peptide competes effectively for binding to FcεRIα receptor in a range comparable with that of unlabeled IgE.

Because neither crystallographic data nor complete nuclear magnetic resonance (NMR) assignments for MCD peptide are available (27), we used molecular modeling to see if any structural differences between MCD peptide and its analog could be detected by this technique. For this purpose Monte Carlo simulations were performed using the PHS approach (20). This technique allows an adequate representation of solvation and extensive sampling of peptide conformations when compared with simulation in a periodic system. The structures generated showed that the connectivities of the two disulfide bonds were possible with the formation of two loops. One big loop included residues 3–15 and a smaller one included residues 3–5 and 16–19. Fig. 2 shows the superimposed structures of the two peptides at the end of one of the respective simulations. It can be seen that these structures are not identical. Most importantly, the distance of the proline residue of MCD peptide to the small loop is shorter than that of the alanine residue in [Ala[12]]MCD, a trend observed in the other simulations as well.

Although the switch for agonism to antagonism usually requires more than one change in the amino acid composition of peptides, the present study shows that a single substitution in the MCD peptide sequence provided an

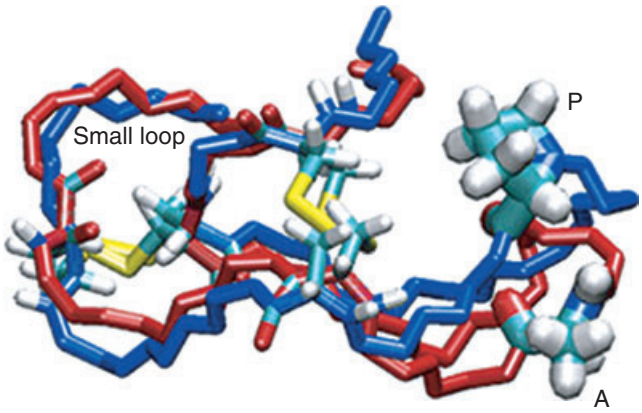

Figure 2. Superimposed structures of mast cell degranulating (MCD) peptide with proline (P) in position 12 and of [Ala[12]]MCD (A) resulted from Monte Carlo simulations. Models were constructed as described under Experimental Procedures.

initial inhibitor for the secretory activity and binding of IgE to the mast cell receptor. Generally, the alanine substitutions influence primarily the side chain peptide–receptor interactions. In this case, however, it seems that the disruption of the *cis–trans* conformational isomerization observed with proline residues in various peptides (28) as well as with MCD peptide (27) leads to conformational rearrangements of the MCD peptide structure (Fig. 2).

It is postulated that there are two binding sites for IgE to the FcεRIα receptor protein (29). Therefore, the structurally altered [Ala[12]]MCD analog might be positioned within or between these two binding sites in a different way than in the parent MCD peptide and thus significantly change its biologic activities.

Acknowledgements: The authors thank Dr S. Masur and R. S. Greenberg, Mount Sinai School of Medicine, for their help with cell culture. Also thank the Heska Corporation (Drs C. Verser and H. Kroona) for the FcεRIα receptor protein.

References

1. Schwartz, L.B. (1994) Mast cells function and contents. *Curr. Opin. Immunol.* **6**, 91–97.
2. Shields, R.L., Wether, W.R., Zioncheck, K., O'Connell, L., Fendly, B., Presta, L.G., Thomas, D., Saban, R. & Jardieu, P. (1995) Inhibition of allergic reactions with antibodies to IgE. *Int. Arch. Allergy Immunol.* **107**, 308–312.
3. McDonell, J.M., Beavil, A.J., Mackay, G.A., Jameson, B.A., Korngold, R., Gould, H.J. & Sutton, B.J. (1996) Structure based design and characterization of peptides that inhibit IgE binding to its high-affinity receptor. *Nat. Struct. Biol.* **3**, 419–426.
4. Helm, B.A., Spivey, A.C. & Padian, E.A. (1997) Peptide blocking of IgE/receptor interactions: possibilities and pitfalls. *Allergy* **52**, 1155–1169.
5. Dahno, W., Makofske, R., Swistok, J., Mallamici, M., Nettleton, M., Madison, V., Greely, D., Fry, D. & Kochan, J. (1999) High affinity IgE receptor α-subunit derived peptides as antagonists of human IgE binding. In: *Peptides: Frontiers of Peptide Science* (Tam, J.P. & Kaumaya, P.T.P., eds), pp. 539–540. Kluwer Academic Publisher, Dordrect.
6. Nakamura, G.R., Starovasnik, M.A., Reynolds, M.E. & Lowman, H.B. (2001) A novel family of hairpin peptides that inhibit IgE activity by binding to the high-affinity IgE receptor. *Biochemistry* **40**, 9828–9835.
7. Habermann, E. (1972) Bee and wasp venoms. *Science* **177**, 314–322.
8. Buku, A. (1999) Mast cell degranulating peptide: a prototypic peptide in allergy and inflammation. *Peptides* **20**, 415–420.
9. Buku, A., Price, J.A., Mendlowitz, M. & Masur, S. (2001) Mast cell degranulating peptide binds to RBL-2H3 mast cell receptors and inhibits IgE binding. *Peptides* **22**, 1993–1998.
10. Buku, A., Reibman, J., Pistelli, A., Blandina, P. & Gazis, D. (1992) Mast cell degranulating peptide analogues with reduced ring structure. *J. Protein Chem.* **11**, 275–280.
11. Buku, A., Mirza, U. & Polewski, K. (1994) Circular dichroism (CD) studies on biological activity of mast cell degranulating (MCD) peptide-mediated histamine release. *Int. J. Pept. Protein Res.* **44**, 410–413.
12. Buku, A., Maulik, G. & Hook, W.A. (1998) Bioactivities on secondary structure of mast cell degranulating (MCD) peptide analogues. *Peptides* **19**, 1–5.
13. Buku, A. & Price, J.A. (2001) Further studies of the structural requirements of mast cell degranulating (MCD) peptide-mediated histamine release. *Peptides* **22**, 1987–1991.
14. Buku, A., Mendlowitz, M., Condie, B.A. & Price, J.A. (2003) Histamine-releasing activity and binding to the FcεRIα human mast cell receptor subunit of mast cell degranulating peptide analogues with alanine substitutions. *J. Med. Chem.* **46**, 3008–3012.
15. Buku, A., Mendlowitz, M., Condie, B.A. & Price, J.A. (2004) Partial alanine scan of mast cell degranulating peptide (MCD): importance of the histidine- and arginine-residues. *J. Pept. Sci.* **10**, 313–317.
16. Barsumian, E.L., Isersky, C., Petrino, M.G. & Siraganian, R.P. (1981) IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur. J. Immunol.* **11**, 317–323.
17. Alber, G., Kent, U.M. & Metzger, H. (1992) Functional comparison of FcεRI, FcεRII, and FcεRIII in mast cells. *J. Immunol.* **149**, 2428–2436.
18. Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S. & Karplus, M. (1983) CHARMM: a program for macromolecular energy minimization and dynamics calculation. *J. Comput. Chem.* **4**, 187–217.
19. Mezei, M (2000) *MMC: Monte Carlo Program for Simulation of Molecular Assemblies*. Available at: http://inka.mssm.edu/~mezei/
20. Kentsis, A., Mezei, M. & Osman, R. (2003) MC-PHS: a Monte Carlo implementation of the primary hydration shell for protein folding and design. *Biophys. J.* **84**, 805–915.
21. Hoffmann, D. & Knapp, E.W. (1996) Polypeptide folding with off-lattice Monte Carlo dynamics: the method. *Eur. J. Biophys.* **24**, 387–403.
22. Mezei, M. (2003) Efficient Monte Carlo sampling for long molecular chains using local moves, tested on a solvated lipid bilayer. *J. Chem. Phys.* **118**, 3874–3880.
23. Jedlovszky, P. & Mezei, M. (1999) Grand canonical ensemble Monte Carlo simulation of a lipid bilayer using extension biased rotations. *J. Chem. Phys.* **111**, 10770–10773.
24. Seldin, D.C., Adelman, S. & Austen, K.F. (1985) Homology of the rat basophilic leukemia cell and the rat mucosal mast cell. *Proc. Natl Acad. Sci. U S A* **92**, 3871–3875.
25. Hakimi, J., Seals, C., Kondas, J.A., Pettine, L., Dahno, W. & Kochan, J. (1990) The α-subunit of the human IgE receptor (FcεRI) is sufficient for high affinity IgE binding. *J. Biol. Chem.* **265**, 22079–22081.
26. Allen, M., Reeves, J. & Mellor, G. (2000) High throughput fluorescence polarization: a homogeneous alternative to radioligand binding for cell surface receptors. *J. Biomol. Screen.* **5**, 63–69.

27. Wayne, E., Bianco, T.I., Zollinger, M. & Perisi, D. (1994) Characterization of the multiple forms of mast cell degranulating peptide by NMR spectroscopy. *Pept. Res.* **7**, 77–82.

28. Balasubramaniam, A., Knittel, J.J., Gil, C. & Andrews, P.C. (1989) Detection of conformational isomers of anglerfish peptide YG (aPY) by reversed phase chromatography. *Int. J. Pept. Protein Res.* **34**, 158–160.

29. Garman, S.C., Wurzburg, B.A., Tarchevskaya, S.S., Kinet, J.-P. & Jardetzky, T.S. (2002) Structure of the Fc fragment of human IgE bound to its high-affinity receptor FcεRIα. *Nature* **406**, 259–266.