Trivalent Antigens for Degranulation of Mast Cells
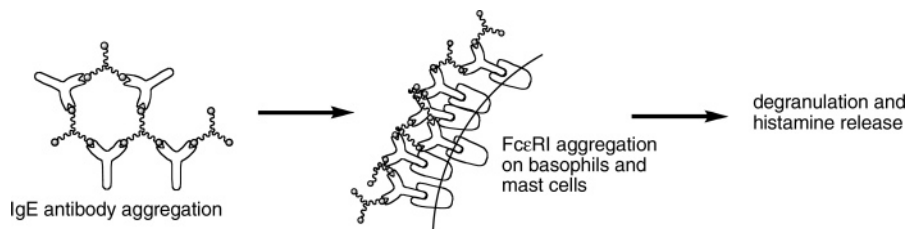
Richard G. Posner,[†,‡] Dianliang Geng,[§] Seth Haymore,[‡] James Bogert,[‡]
Israel Pecht,[#] Arieh Licht,[#] and Paul B. Savage*,[§]

Department of Computational Biology, Translational Genomics Research Institute, Phoenix, Arizona 85004, Department of Biology, Northern Arizona University, Flagstaff, Arizona 86011, Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602, and Department of Immunology, Weizmann Institute of Science, Rehovet 76100, Israel

paul_savage@byu.edu

ABSTRACT

IgE antibody aggregation → FcεRI aggregation on basophils and mast cells → degranulation and histamine release

Degranulation of basophils and mast cells plays a central role in allergic reactions. Degranulation is a response to cell surface receptor aggregation caused by association of receptors with antibodies bound to multivalent antigens. Tools used in studying this process have included small-molecule divalent antigens, but they suffer from weak signaling apparently due to small aggregate size. We have prepared trivalent antigens that allow formation of larger aggregates and potent responses from mast cells.

Aggregation of cell surface receptors is a common mechanism involved in signal transduction across cell membranes.[1] This mechanism is used, for example, by receptors that are intrinsic protein tyrosine kinases (i.e., autophosphorylation of aggregated receptors),[2,3] such as the epidermal growth factor receptor, the platelet derived growth factor receptor, and the high affinity receptor for IgE (FcεRI), which plays a central role in allergic reactions.[4,5] Early steps in the initiation of signaling by this class of receptors are similar: multivalent interactions with a ligand lead to aggregation of receptors and enhanced phosphorylation of tyrosines, which can be recognized by cytoplasmic regulatory molecules.

FcεRI are found on the surface of mast cells, basophils, and rat basophilic leukemia (RBL) cells, and these receptors bind the Fc (invariant) region of divalent IgE antibodies. When multiple IgE molecules bind a polyvalent antigen and in turn associate with FcεRI on a cell surface, aggregates of these receptors are generated (Figure 1). Formation of aggregates can in some cases trigger secretion of inflammatory mediators.

Signals generated by FcεRI aggregation depend on various properties of the aggregate structures that are formed on the cell surface including the size of aggregates,[6] the spacing of receptors in aggregates,[7] and the time that individual receptors spend within a complex.[8]

† Translational Genomics Research Institute.
‡ Northern Arizona University.
§ Brigham Young University.
# Weizmann Institute of Science.
(1) Metzger, H. *J. Immunol.* **1992**, *149*, 1477.
(2) Pazin, M. J.; Williams, L. T. *Trends Biochem. Sci.* **1992**, *17*, 374.
(3) Fry, M. J.; Panayotou, G.; Booker, G. W.; Waterfield, M. D. *Protein Sci.* **1993**, *2*, 1785.
(4) Keegan, A. D.; Paul, W. E. *Immunol. Today* **1992**, *13*, 63.
(5) Holowka, D.; Baird, B. *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 79.

(6) Ortega, E.; Schweitzer-Stenner, R.; Pecht, I. *EMBO J.* **1988**, *7*, 4101.
(7) Kane, P.; Ericksion, J.; Fewtrell, C.; Baird, B.; Holowka, D. *Mol. Immunol.* **1986**, *23*, 783.
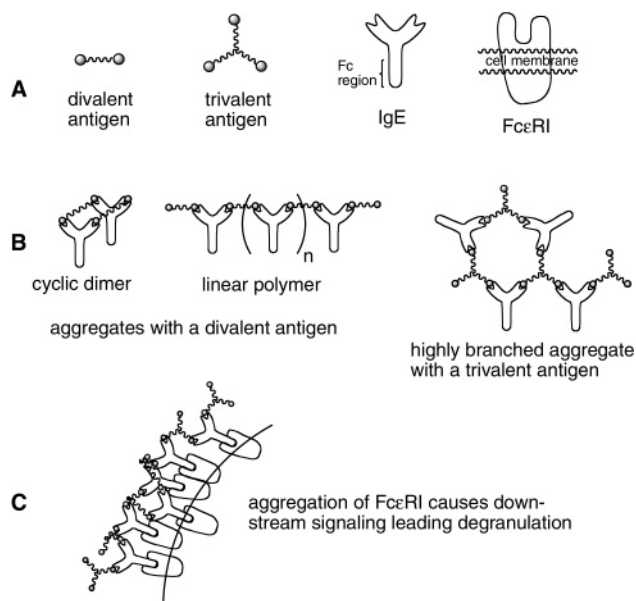(8) Schweitzer-Stenner, R.; Pecht, I. *Immunol. Lett.* **1999**, *68*, 59.

Figure 1. (A) Schematic representation of ligands, antibodies and receptors involved in degranulation; (B) IgE aggregates formed with di- and trivalent antigens; (C) aggregation of FcεRI leading to aggregation.

For example, it has been observed that aggregation caused by IgE dimers are less effective than those caused by larger IgE oligomers at stimulating cellular responses[6] and that cellular responses are inhibited when an optimal degree of aggregation is exceeded.[9] The factors that cause an aggregate to be a robust signaling unit, an inhibitor of signal transduction, or a nonsignaling unit remain to be fully elucidated.

The importance of influences of ligand-induced receptor aggregates on cellular signals has prompted significant effort in developing means for quantitative analysis of the interactions between multivalent ligands and cell surface receptors.[10] An attractive approach to studying IgE−FcεRI aggregation involves the use of synthetic symmetric divalent ligands, which are the simplest type of ligand capable of aggregating receptors. The interaction of divalent IgE antibodies with a divalent ligand can result in an IgE−FcεRI aggregate spectrum that contains only linear chains or rings of various sizes (including one-to-one complexes) (Figure 1B). This limited spectrum of aggregates makes divalent ligands much easier to study theoretically than ligands of valences higher than two, which can form highly branched aggregates. A primary goal of studies performed with divalent ligands has been to measure or predict the number of receptors in aggregates on the cell surface, so that this quantity then can be compared and correlated with cellular responses.[11] However, a major limitation of divalent ligands studied to date is their inability to activate strong cellular responses.

(9) Seagrave, J.; Oliver, J. M. *J. Cell Physiol.* **1990**, *144*, 128.

(10) Hlavacek, W. S.; Perelson, A. S.; Sulzer, B.; Bold, J.; Paar, J.; Gorman, W.; Posner, R. G. *Biophys. J.* **1999**, *76*, 2421.

(11) Posner, R. G.; Subramanian, K.; Feder, T.; Thomas, J.; Holowka, D.; Baird, B.; Goldstein, B. *J. Immunol.* **1995**, *155*, 3601.

Evidence suggests that these ligands are poor initiators of cell signaling because they aggregate receptors predominantly in the form of stable cyclic dimers.[12] Modeling suggests that cyclic dimers, in which two ligand molecules connect two IgE−FcεRI complexes to form a closed ring, prevent chain elongation, limit the size of ligand-induced aggregates, and may generate an inhibitory signal.[13]

In contrast to divalent antigens, trivalent antigens are predicted to form larger, highly branched aggregates (Figure 1B) leading to strong responses by IgE−FcεRI presenting cells (Figure 1C). Recently, Whitesides and co-workers[14] reported the preparation of a trivalent antigen capable of aggregating IgE. They found that aggregates formed with stoichiometries of 3:2 (IgE/trivalent ligand). In this manuscript we describe the synthesis of a series of di- and trivalent ligands and comparison of their relative ability to stimulate degranulation in IgE sensitized RBL cells. We find that the trivalent ligands are potent antigens causing degranulation at nanomolar concentrations, while analogous divalent antigens are inactive by comparison.
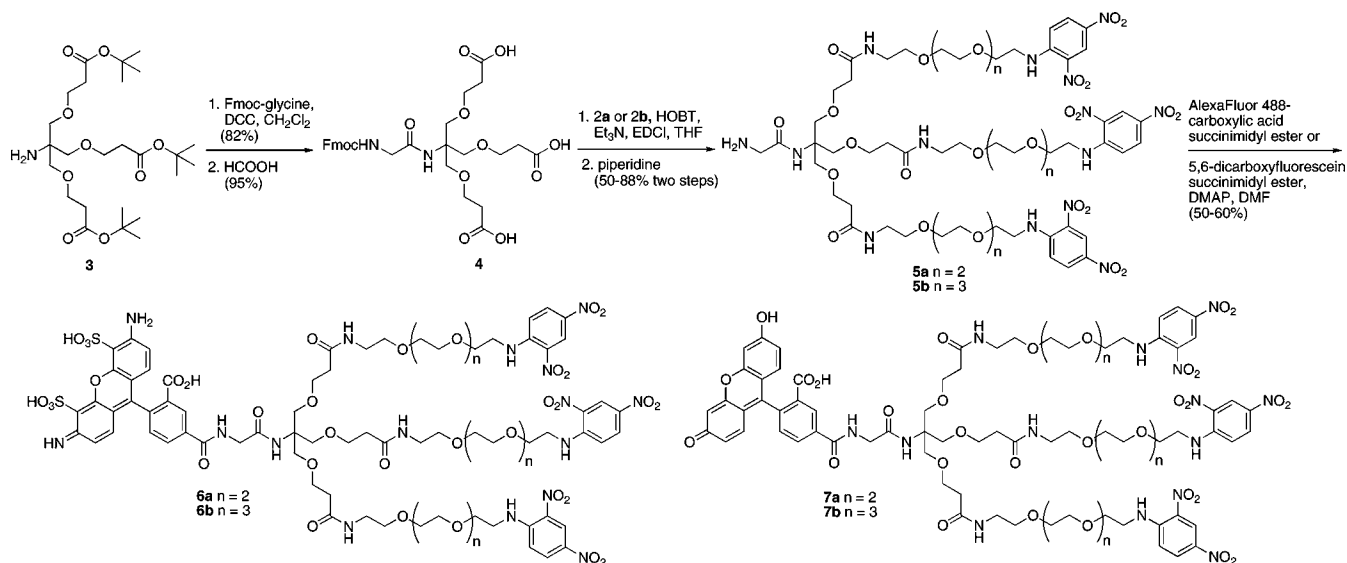
In allergic responses resulting in release of inflammatory mediators, IgE targeting a nearly limitless variety of antigens may be involved. To simplify experiments, monoclonal IgE that associates with dinitrophenyl groups is used, and multivalent antigens therefore contain multiple dinitrophenyl amines. RBL cells are readily culturable and respond strongly to receptor aggregation and consequently are useful in measuring responses to polyvalent antigens. Measurement of degranulation is made by spectroscopic determination of the activity of granule-stored β-hexosaminidase secreted into the supernatant on *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine.

Design considerations of the multivalent antigens included the length of the tether between dinitrophenylamine groups and an attachment point for an additional fluorophore. In earlier work, we determined that separation of antigenic epitopes of more than 40 Å was sufficient to allow efficient binding to IgE and subsequent aggregation of FcεRI.[15]

To ensure that a tether of adequate length was generated, we prepared tethers derived from both tetra- and pentaethylene glycol. On the basis of the modeling of the tethers (MMFF with Spartan) a maximum separation of 43.3 and 49.5 Å between dinitrophenylamine groups was predicted for the tetraethylene and pentaethylene glycol-based compounds, respectively. The tethers were prepared using Schwabacher's effective desymmetrization procedure of a diazide[16] giving **1a** and **1b** (Scheme 1), followed by reaction with fluorodinitrobenzene and reduction providing **2a** and **2b**.

Design of trivalent antigens was based on a tris-hydroxymethylaminomethane (Tris) core because this com-

Scheme 1. Preparation of Dinitrophenyl-Appended Glycol Linkers.
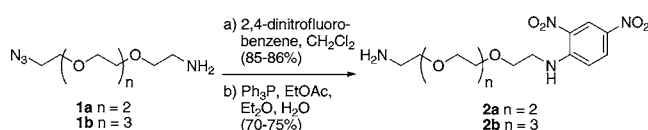
Scheme 2. Preparation of Fluorophore-Appended Trivalent Antigens.

Figure 2. Structures of divalent antigens **8a** and **8b**.

pound allows rapid generation of trivalent antigens. In addition, the amine group in Tris provides a reactive group for attachment of labels including fluorophores that can be included to allow fluorescent observation of binding events. The Tris core has been used by multiple groups for the generation of dendrimers and other multivalent compounds,[17,18] and the initial step in extending groups from Tris typically involves 1−4 addition to acrylates.[17] We used *t*-butyl acrylate appended Tris (**3** in Scheme 2). Reaction of the free amine in **3**[18] with activated Fmoc-glycine and unmasking of the three acid groups gave **4**. Attachment of the tethered dinitrophenylamines through amide bonds was followed by removal of the Fmoc protecting group yielding **5a** and **5b**. Reaction with succinimidyl esters of Alexa-488 or fluorescein gave the series of labeled trivalent antigens **6a**, **6b** and **7a**, **7b**.

To compare the differences between divalent antigens capable of generating only linear and cyclic aggregates and trivalent antigens capable of generating highly branched aggregates, divalent antigens **8a** and **8b** (Figure 2) were prepared using **2a** and **2b** and serinol as a core. The methodology described in Scheme 2 was used for preparation of these compounds (see Supporting Information).

The abilities of each of the tri- and divalent antigens to stimulate degranulation were measured using the enzymatic

assay described above. The results are shown in Figure 3. All assays were repeated at least four times and their results are expressed as net percent of the cells' total enzyme activity contents ±5 to 10%.
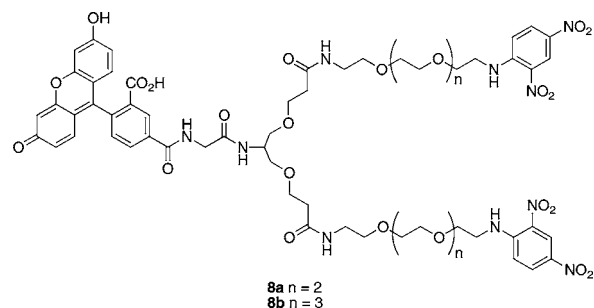
Trivalent antigens **6a**, **6b**, **7a**, and **7b** all proved to be very potent stimulators of RBL cells, with degranulation occurring in response to concentrations of the antigens below the nM level. The differences in stimulation between the trivalent antigens with Alexa-488 and those with fluorescein may be attributable to the higher solubility of Alexa-488, which may decrease self-aggregation. The tether lengths exerted a modest influence on the level of degranulation with longer tether lengths (**6b** and **7b**) providing greater degranulation than the antigens with shorter tether lengths.

It is significant that above a threshold concentration of the trivalent antigens, degranulation drops off. Aggregation increases with ligand concentration up to an optimal ligand concentration and then decreases as monovalent binding, because of excess ligand, begins to predominate. For any polyvalent ligand interacting with a bivalent recepor (such as IgE), one expects that the aggregation curve, that is, a

(12) Schweitzer-Stenner, R.; Licht, A.; Pecht, I. *Biophys. J.* **1992**, *63*, 563.

(13) Harris, N. T.; Goldstein, B.; Holowka, D.; Baird, B. *Biochemistry* **1997**, *36*, 2237.

(14) Bilgicer, B.; Moustakas, D.; Whitesides, G. M. *J. Am. Chem. Soc.* **2007**, *129*, 3722.

(15) Posner, R. G.; Savage, P. B.; Peters, A. S.; Macias, A.; DelGado, J.; Schwarz, G.; Sklar, L. A.; Hlavacek, W.; Goldstein, B. *Mol. Immunol.* **2002**, *38*, 1221.

(16) Schwabacher, A. W.; Lane, J. W.; Schiesher, M. W.; Leigh, K. M.; Johnson, C. W. *J. Org. Chem.* **1998**, *63*, 1727.

(17) For examples see: (a) Rensen, P. C. N.; van Leeuwen, S. H.; Sliedregt, L. A. J. M.; Van Berkel, T. J. C.; Biessen, E. A. *J. Med. Chem.* **2004**, *47*, 5798. (b) Kawa, M.; Takahagi, T. *J. Polym. Sci., Part B: Polym. Phys.* **2004**, *42*, 2680.

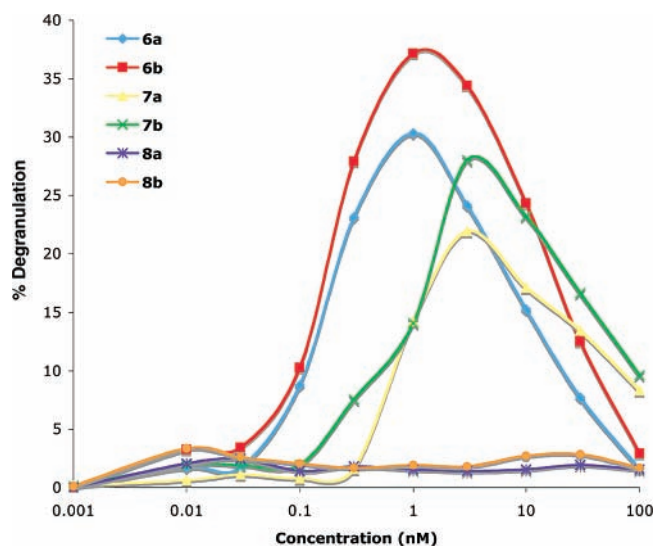(18) Claudia, M. C.; Robert, E. G. *J. Org. Chem.* **2002**, *67*, 1411.

Figure 3. Degranulation of mast cells (RBL-2H3 cells) in response to di- and trivalent antigens as measured by secreted β-hexosaminidase activity.

plot of the degree of aggregation versus the concentration of ligand, is bell shaped.

Results with **8a** and **8b** are notable because over the concentration range shown, no degranulation above background was observed. Because the divalent ligand and trivalent antigens are expected to have similar binding affinities between their dinitrophenylamine groups and the IgE binding sites and the spacing between dinitrophenylamines are the same, it seems likely that the differences in

cellular response occur because aggregates formed by the trivalent antigens are not simple "cyclic dimers" but rather more complex networks and branched structures (Figure 1). Considering this concentration range and the concentration at which the trivalent antigens begin to provide signaling for degranulation, it is apparent that the trivalent antigens are at least 1000 times better at generating aggregates that provide a positive signal.

In conclusion, degranulation in response to the trivalent antigens is notable because these symmetric trivalent antigens are the first (and simplest) well-defined, synthetic ligands produced to date that generate the same type of strong cellular signals that one observes with highly polyvalent antigens such as conjugated proteins (polydinitrophenyl-bovine serum albumin,[13] polydinitrophenyl-polyethylene glycol[19]) in this cell system. It is anticipated that these small, potent, fluorophore-labeled antigens will facilitate study of receptor aggregation on cell surfaces and signaling that leads to allergic responses.

Supporting Information Available: Experimental details for the degranulation experiments; a synthetic scheme for **8a** and **8b**; synthetic details for the preparation of the di- and trivalent antigens (**6a,b**, **7a,b**, **8a,b**); and ^{1}H NMR spectra for **6a,b**, **7a,b**, **8a,b**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL071175H

(19) Baird, E. J.; Holowka, D.; Coates, G. W.; Baird, B. *Biochemistry* **2003**, *42*, 12739.