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TESIS DOCTORAL

ESTABLISHMENT OF AN IN VITRO MAST CELL RAPID DESENSITIZATION PROTOCOL AND ELUCIDATION OF ITS MECHANISM FOR IgE-DEPENDENT ANTIGENS

Presentada por

María del Carmen Sancho Serra

para optar al título de Doctora por la Universidad de Granada

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***Dra. ANA ABADÍA MOLINA**, Profesora Titular de Inmunología de la Universidad de Granada y **Dra. MARÍA CONCEPCIÓN CASTELLS GUITART**, Associate Professor en la Universidad de Harvard y Directora de la Unidad de Desensibilización del Departamento de Reumatología, Inmunología y Alergia del Brigham and Women's Hospital y Directora del Programa de formación de Alergia e Inmunología Clínica del Brigham and Women's Hospital, en Boston.*

CERTIFICAN:

Que el presente trabajo de investigación presentado por María del Carmen Sancho Serra, titulado "ESTABLISHMENT OF AN IN VITRO MAST CELL RAPID DESENSITIZATION PROTOCOL AND ELUCIDATION OF ITS MECHANISM FOR IgE-DEPENDENT ANTIGENS", y realizado bajo nuestra dirección, reúne las condiciones necesarias para ser defendido como Tesis Doctoral ante el tribunal correspondiente.

Y para que conste a los efectos oportunos, expedimos y firmamos el presente certificado en Granada, a 16 de septiembre de 2011.

Fdo. Ana Abadía Molina *Fdo. María Concepción Castells Guitart*

a Lola i a Xavi…
…els dos meus amors

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The conception that antibodies, which should protect against disease, are also responsible for disease, sounds at first absurd

Clemens von Pirquet (1906)

50 Euro gold coin
"Begründer der Allergielehre" (Founder of the science of allergies)

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ABBREVIATIONS

12-HHT: 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid
BMMC: bone marrow derived mast cell
BSA: bovine serum albumin
CO2: carbon dioxide
COX: cyclooxygenase
CRAC: Ca^{2+} release-activated Ca^{2+} (CRAC) channels
DAPI: 40,6-diamidino-2-phenylindole dihydrochloride
DME: Dulbecco's modified Eagle's medium
DNA: deoxyribonucleic acid
DNP-HSA: 2,4-Dinitrophenyl HSA-conjugated
DTT: dithiothreitol
EGTA: ethyleneglycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinase
FACS: fluorescence-activated cell sorter
FBS: fetal bovine serum
FCS: fetal calf serum
FITC: Fluorescein isothiocyanate
Fura: 2-AM fura 2-acetoxymethyl ester
HBSS: Hanks' balanced salt solution
HSA: globulin free human serum albumin
Ig: immunoglobulin
IL: Interleukin
IL: interleukin (e.g., IL-3)
i.p.: intra peritoneal
IP3: inositol-1,4,5-trisphosphate
ITAM: Immunoreceptor Tyrosine-based Activation Motif
ITIM: Immunoreceptor Tyrosine-based Inhibitory Motif
kD: kilo Dalton
KO: Knockout
LAT: linker for the activation of T cells
LTB4: leukotriene B4
LTC4: cysteinyl leukotriene C4
MAPK: mitogen-activated protein kinase
MFI: mean fluorescence intensity
MHC: major histocompatibility complex
Na3VO4: sodium orthovanadate
NaCL: sodium chloride
NaF: sodium fluoride
NFAT: nuclear factor of activated T cells
NP-40: Nonidet P-40
OVA: ovalbumin
PBS: phosphate-buffered saline

PE: phycoerythrin
PG: prostaglandin
PIP2: phosphatidylinositol-4,5-bisphosphate
PI3K: phosphatidylinositol 3-kinase
PMSF: phenylmethylsulfonyl fluoride
R: receptor (e.g., IL-4R)
RBL-2H3: rat basophilic leukemia cell line
RP-HPLC: reverse-phase high-performance liquid chromatography
RPMI: Roswell Park Memorial Institute media
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: standard error of the mean
SHIP: Src homology 2 domain-containing inositol 5' phosphatase
SHP: Src homology 2 domain-containing tyrosine phosphatase
STAT: signal transducer and activator of transcription (e.g., STAT6)
TBST: Tris Buffered Saline (TBS) with Tween 20
TNF: tumor necrosis factor
TNP: trinitrophenyl
Tris: tris (hydroxymethyl) aminomethane
UV: ultraviolet
WT: wild-type

SUMMARY

Mast cells are immune effector cells that can store a wide range of inflammatory mediators in their granules, with important roles in innate and adaptive immunity. Mast cell activation can be triggered by the crosslinking of two or more high affinity receptors for IgE (FcεRI) by IgE and antigen/allergen binding to the alpha subunit, which induces the aggregation of the receptors and transphosphorylation of the beta and gamma subunits. This activation triggers the release of powerful mediators such as histamine, proteases, proteoglycans, prostaglandins and leukotrienes among others. These mediators act upon local and systemic tissue receptors and induce symptoms such as, flushing, puritus, hives, angioedema, bronchoconstriccion, diarrhea, vomiting, hypotension and cardiovascular collapse, which can lead to death in a few minutes (anaphylaxis) in patients sensitized to drugs, foods or environmental allergens (Schwartz LB et al. / Vadas P et al.).

IgE-mediated mast cell activation through the FcεRI, during type I hypersensivity reactions, has been implicated in diseases such as asthma, rhinitis and drug and food allergies and anaphylaxis. The prevalence of allergic diseases has been increasing the last 30 years in developed countries with poor exposure to the sun (Raby BA et al.) and parasites (hygiene hypothesis) (von Mutius E). Reactions to essential drugs are also increasing because patients live longer and better due to better treatment options and targeted therapies. Thus prolonged exposure to the drugs induces sensitization in a significant proportion of this patient's population (Brennan PJ et al.). For patients sensitized to a first line medication for the treatment of cancer or a serious infection, the allergy to such drug may increase the morbidity and reduce their life span (Navo M et al.). For patients sensitized to certain foods, allergic reactions can occur when eating cross-contaminated or cross-reactive foods even in patients who do consistent avoidance of the offending foods.

To overcome avoidance for the patients in need of first line therapy, rapid drug desensitization protocols have been generated for type I hypersensitivity reactions. These protocols have become an essential tool which allows the delivery of therapeutic doses of the offending drug in a relatively short time and in an effective and safe way with minimal risk for the patients (Lee CW et al. (1) / Lee CW et al. (2) / Castells MC et al. (3) / Legere HJ 3rd et al.).

IgE sensitized patients present positive skin test to the specific drug implicated in the reaction, which indicates that mast cells and IgE are the main cellular and molecular targets implicated in allergic reactions. After rapid drug desensitization, the specific skin test becomes negative, which demonstrates a profound inhibition of mast cell activation (Lee CW et al. (1)). Because mechanisms underlying the inhibition of mast cell responses are not completely understood and due to its clinical importance and relevance, it is critical to identify the cellular and molecular mechanisms behind the temporary tolerance induced by rapid desensitization.

Initial studies of *in vitro* mouse mast cell desensitizations showed that incubation of mast cells with non-activating antigen doses and in the absence of

calcium, induces the inhibition of the activation with optimal doses once the calcium is re-introduced (Ishizaka T et al.). Similar results were obtained in human basophils, inducing inhibition of cellular activation with repetitive suboptimal doses of antigen or anti-IgE added at regular intervals or for a prolonged period of time (Mendoza GR et al. (1) / Mendoza GR et al. (2)). But calcium-free conditions cannot possibly be applied to human desensitizations. Few studies have undertaken physiological desensitizations of mast cells (Shalit M et al.) and basophils examining their releasability following hours to days of culture with low levels of antigen or anti-IgE antibody in calcium-containing medium, resulting in full desensitization (Kepley CL (2) / Komiya A et al.).

In this PhD thesis, we propose a mouse mast cell rapid desensitization protocol in physiological calcium conditions. We study and analize the desensitization process, characterizing its kinetics and proving the reproducibility, the effectiveness and the versatility of the protocol. This protocol uses two important allergens: dinitrophenyl (DNP), which has been validated in a previous study (Morales AR et al.) and ovalbumin (OVA), moreover, the protocol can be used for murine bone marrow mast cells (m-BMMCs) and a rat basophilic leukemia cell line (RBL-2H3) and it can be adapted to different target doses (1, 5 and 10 ng for DNP and 10 and 50 ng for OVA). Desensitized mast cells show an almost complete inhibition of degranulation, calcium flux, arachidonic acid metabolism for prostaglandin and leukotriene generation, IL-6 and TNF-α synthesis and STAT6, LAT and p38 MAPK phosphorylation. Thus, rapid desensitization inhibits all hallmarks of mast cell activation.

The model provides insight into the specificity of the rapid desensitization process since DNP desensitized mast cells fully respond to OVA and vice versa. Even more importantly, we show that the mechanism of impaired activation is due to the luck of internalization of the antigen/IgE/FcεRI complex. This model for the first time offers an initial mechanistic approach to rapid desensitizations and validates the safety of human desensitizations. Furthermore, this study may constitute an important tool for the development of improved and safer protocols for drug and food desensitizations.

RESUMEN

Los mastocitos son células efectoras del sistema inmunitario que poseen la habilidad de almacenar un amplio rango de mediadores inflamatorios en los gránulos, con un papel relevante en la inmunidad innata y en la adquirida. La activación del mastocito se produce cuando la inmunoglobulina E (IgE) y el antígeno/alérgeno se unen a la subunidad alfa de dos o más receptores de elevada afinidad por la IgE (FcεRI) provocando el entrecruzamiento y la agregación de dichos receptores y la transfosforilación de sus subunidades beta y gamma. Esta activación provoca la liberación de potentes mediadores tales como la histamina, proteasas, proteoglicanos, prostaglandinas y leucotrienos entre otros. Estos mediadores actúan en receptores de tejidos de forma local y sistémica induciendo síntomas como enrojecimiento, prurito, urticaria, angioedema, broncoconstricción, diarrea, vómito, hipotensión y colapso cardiovascular, lo que puede conllevar la muerte en pocos minutos (anafilaxia) en pacientes sensibilizados a medicamentos, alimentos o alérgenos ambientales (Schwartz LB et al. / Vadas P et al.).

La activación del mastocito mediada por IgE a través del FcεRI, en las reacciones de hipersensibilidad de tipo I, ha sido implicada en enfermedades tales como asma, rinitis, alergia a medicamentos y alimentos y anafilaxia. La prevalencia de enfermedades alérgicas ha ido creciendo en los últimos 30 años en países desarrollados con poca exposición al sol (Raby BA et al.) y a parásitos (hipótesis de la higiene) (von Mutius E). Reacciones a medicamentos esenciales van también en aumento porque los pacientes viven más y mejor debido a mejores opciones de tratamiento y a terapias diana. Es por ello que la exposición prolongada a medicamentos induce sensibilización en una proporción significativa en dicha población de pacientes (Brennan PJ et al.). Para pacientes sensibilizados a un medicamento de primera necesidad para el tratamiento de cáncer o de una infección grave, la alergia a dicho medicamento puede incrementar la morbosidad y reducir su tiempo de vida (Navo M et al.). Para pacientes sensibilizados a ciertos alimentos, las reacciones alérgicas pueden ocurrir al ingerir un alimento contaminado con otro de reactividad cruzada incluso en pacientes que evitan de forma consistente los alimentos alergénicos.

En el caso de pacientes alérgicos a un medicamento de primera necesidad y para superar el rechazo, se han generado protocolos de desensibilización rápida a medicamentos para reacciones de hipersensibilidad de tipo I. Dichos protocolos se han convertido en una herramienta esencial que permite el suministro de dosis terapéuticas del medicamento alergénico en un tiempo relativamente corto y de manera efectiva y segura con un riesgo mínimo para los pacientes (Lee CW et al. (1) / Lee CW et al. (2) / Castells MC et al. (3) / Legere HJ 3rd et al.).

Pacientes con sensibilización mediada por IgE presentan una prueba cutánea positiva al medicamento específico implicado en la reacción, lo que indica que los mastocitos y la IgE son las principales células y moléculas clave implicadas en las reacciones alérgicas. Una vez finalizada la desensibilización rápida al medicamento, la prueba cutánea aparece negativa, lo que demuestra

una enorme inhibición de la activación del mastocito (Lee CW et al. (1)). Debido a que los mecanismos subyacentes en la inhibición de la respuesta del mastocito no están completamente entendidos y debido a su relevancia e importancia clínica, es fundamental identificar los mecanismos celulares y moleculares que hay detrás de la tolerancia temporal inducida por la desensibilización rápida.

Estudios iniciales de desensibilizaciones de mastocitos de ratón *in vitro* han mostrado que la incubación de mastocitos con dosis no activadoras de antígeno y en ausencia de calcio, inducen la inhibición de la activación con dosis óptimas una vez el calcio es reintroducido (Ishizaka T et al.). Resultados similares fueron obtenidos en basófilos humanos, induciendo la inhibición de la activación celular con dosis subóptimas repetitivas de antígeno o de anti-IgE adicionadas a intervalos regulares o durante un largo periodo de tiempo (Mendoza GR et al. (1) / Mendoza GR et al. (2)). Sin embargo, condiciones sin calcio no pueden ser aplicadas a desensibilizaciones humanas. Algunos estudios de desensibilizaciones fisiológicas se han llevado a cabo con mastocitos (Shalit M et al.) y con basófilos examinando su capacidad de degranulación después de horas y días de cultivo con bajos niveles de antígeno o de anticuerpos anti-IgE en un medio con calcio, dando como resultado una desensibilización completa (Kepley CL(2) / Komiya A et al.).

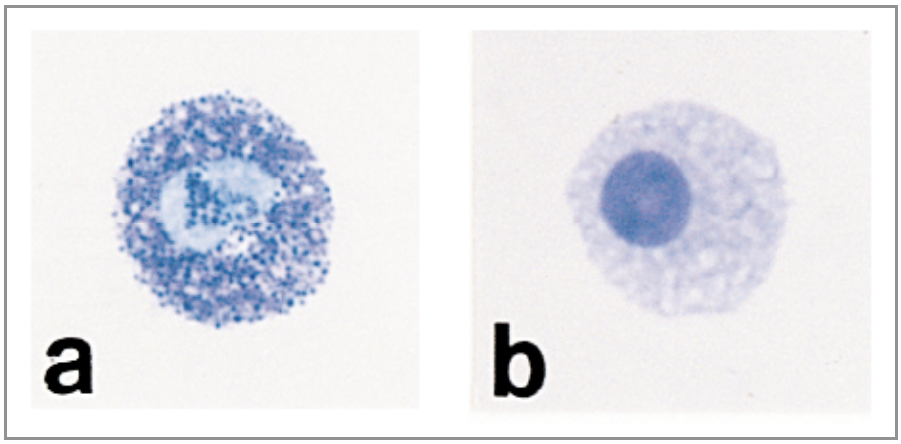
En esta tesis, proponemos un protocolo de desensibilización rápida de mastocitos de ratón en condiciones de calcio fisiológicas. Estudiamos y analizamos el proceso de desensibilización, caracterizando su cinética y probando la reproducibilidad, efectividad y versatilidad del protocolo. Dicho protocolo utiliza dos alérgenos importantes: dinitrofenol (DNP), que ya ha sido validado en un estudio previo (Morales AR et al.) y ovalbúmina (OVA), además, el protocolo puede ser utilizado con mastocitos derivados de médula osea de ratón (m-BMMCs, mouse Bone Marrow Mast Cells) y con la línea celular mastocitaria RBL-2H3 (Rat Basophilic Leukemia-Leucemia Basofílica de Rata) y puede ser adaptado a diversas dosis objetivo (1, 5 y 10 ng para DNP y 10 y 50 ng para OVA). Los mastocitos desensibilizados muestran una inhibición casi completa de la degranulación, del flujo de calcio, del metabolismo del ácido araquidónico para la generación de prostaglandinas y leucotrienos, de la síntesis de IL-6 y de TNF-α y de la fosforilación de STAT6, LAT y p38 MAPK. Así pues, la desensibilización rápida inhibe todas las funciones características de la activación mastocitaria.

El modelo proporciona una percepción de la especificidad del proceso de desensibilización rápida ya que mastocitos desensibilizados a DNP responden completamente a OVA y viceversa. Aún más importante, mostramos que el mecanismo de la anulación de la activación se debe a la ausencia de internalización del complejo antígeno/IgE/FcεRI. Este modelo, por primera vez ofrece una aproximación inicial al mecanismo de la desensibilización rápida y valida la seguridad de las desensibilizaciones humanas. Además, este estudio puede proporcionar una herramienta para la mejora y mayor seguridad de las desensibilizaciones a medicamentos y a alimentos.

INTRODUCTION

1. Mast cell biology and relevance

Mast cells were first described by Paul Ehrlich in his 1878 doctoral thesis and he named them "*Mastzellen*" (fattened cells) because they contain granules rich in acidic proteoglycans that confers them a unique staining characteristics with basic dyes such as Toluidine blue (***Figure 1***).



Heavy Toluidine Blue **Light Toluidine Blue**

Figure 1. *Mast cells stained with Toluidine blue, one of the most common stains for acid mucopolysaccarides and glycoaminoglycans, components of mast cells granules.* ***Adapted from Gurish MF et al.***

The origin of these cells, however, remained obscure for many years. It is now accepted that mast cells arise from pluripotential hematopoietic cells in the bone marrow (Sonoda T et al.) and then enter the circulation in an immature form. Once settled into a tissue site, they mature, taking on characteristics specific for that tissue.

Their heterogeneity has been described in mice and humans, based on the protease content of the granules. Thus, mast cells found in connective tissue differ than those found in mucosal tissues. In most cases, mast cells are strategically located around blood vessels, in the lining of all mucosal tissues and in the connective tissues. They are also present in places exposed to the external environment such as the skin (***Figure 2***) but especially the mucosa of the lungs, digestive tract, mouth, nose, and eyes (Marshall JS).

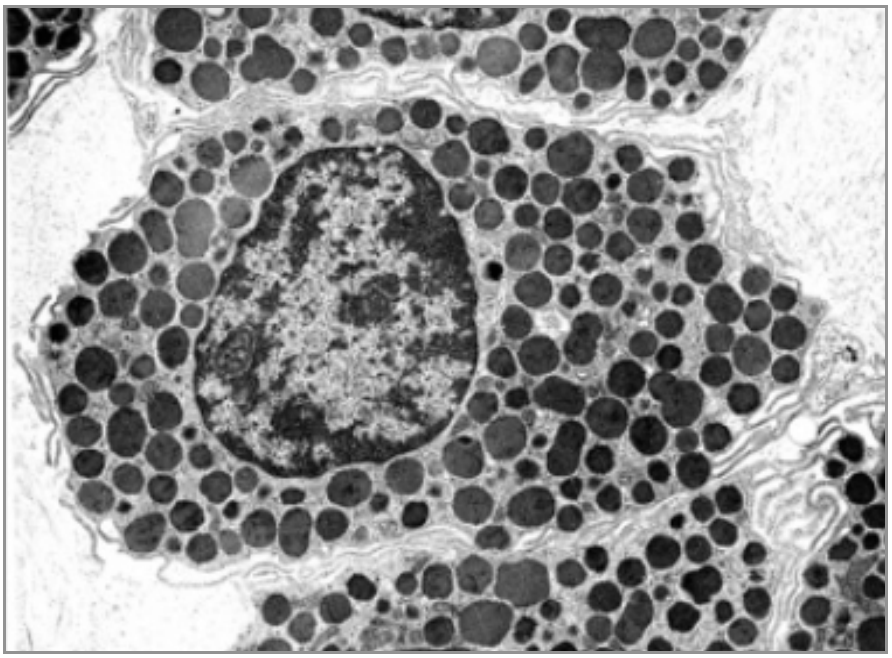


Figure 2. *Transmission electron microscopy image of a skin mast cell. Mature mast cell with a cytoplasm full of secretory granules and few cytoplasmic organelles, with the nucleus in the center.* ***Courtesy of Mariana Castells***

Mast cells participate in the innate and acquired immune response and play a key role in many immunological and inflammatory reactions such as asthma, rhinitis, food allergies, anaphylaxis, rheumatoid arthritis, and other autoimmune diseases. A beneficial role for these cells has been described in defense against bacteria, virus and parasites (Galli SJ et al. (1)). They are, however, best known as the critical effector cells in immunoglobulin E-associated allergic disorders.

The manifestations of mast cell-driven allergic reactions are the consequence of the release of an array of pro-inflammatory mediators. These mediators include pre-formed granule-associated components, newly generated membrane-derived lipid mediators, and newly synthesized cytokines that are generated and released over several hours (Blank U et al.).

2. Mast cell receptors

The ultimate response of a cell to its environment is determined by the balance of stimulatory and inhibitory factors present at a given moment and acting on different receptors. Mast cells appear to be highly regulated cells with multiple critical biological functions, displaying a host of stimulatory and inhibitory surface receptors that allow them to respond to a variety of stimuli.

2.1. Activating receptors

The most important stimulatory receptors on the surface of mast cells include the high affinity IgE receptor (FcεRI), receptors for stem cell factor (c-Kit), IgG receptors (FcγRI or CD64 and FcγRIII or CD16), toll-like receptors and complement proteins. Many of the activating receptors contain immuno-receptor tyrosine-based activation motifs (ITAMs) that are crucial in the generation of the activating signal.

Receptor c-Kit is a single chain receptor with intrinsic tyrosine kinase activity. The extracellular domain possesses five immunoglobulin-like regions that contain the binding site for its ligand, stem cell factor (SCF). Within the cytosolic tail, there is a split tyrosine kinase catalytic domain and multiple tyrosine residues that serve as auto-phosphorylation sites after kit activation. These phosphorylated sites subsequently recruit specific signaling molecules that are crucial for kit-mediated responses (Roskoski R Jr).

The high affinity FcεRI is a multi-chain receptor complex consisting of an α chain, a β chain and a γ chain homodimer. FcεRI, a most important receptor for IgE-mediated mast cell activation, is described below in point 3.

2.2. Inhibitory receptors

Immune responses are crutial to fight pathogensMast cells express several inhibitory receptors that have been shown to regulate mast cell-mediated events and mast cell-dependent inflammation. Many of the inhibitory receptors contain immuno-regulatory tyrosine inhibition motifs (ITIMs).

Examples of ITIM-associated receptors capable of suppressing mast cell activation are FcγRII-B1 or CD32, CD300a, platelet-endothelial cell adhesion molecule 1 (PECAM-1), paired immunoglobulin-like receptor B (PIR-B), the c-lectin mast cell function-associated antigen (MAFA) and Leukocyte Immunoglobulin-like receptor B4 (LILRB4) also know as glycoprotein 49B1 (gp49B1).

Antibody-mediated colligation of gp49B1 with FcεRI on mast cells *in vitro* inhibits the release of secretory granule mediators such as histamine and β-hexosaminidase, as well as suppressing the generation and secretion of the lipid mediator, leukotriene C4 (Katz HR. (3) / Castells MC et al. (2) / Lu-Kuo JM et al. / Katz HR. et al. (1) / Katz HR. et al. (2)).

3. Mast cell activation via the FcεRI

Mast cell activation requires coordinated events in order to respond to an allergen and depends on IgE and its high affinity receptor for IgE (FcεRI) present on the surface of these cells.

3.1 FcεRI structure

The FcεRI is a multimeric cell-surface receptor that binds the Fc fragment of the IgE with high affinity. In humans, it exists as a tetrameric form on mast cells and basophils and as a trimeric form on antigen-presenting cells. Murine FcεRI exists just as a tetramer and it is only present in mast cells and basophils (Kraft S et al. / Abramson J et al.).

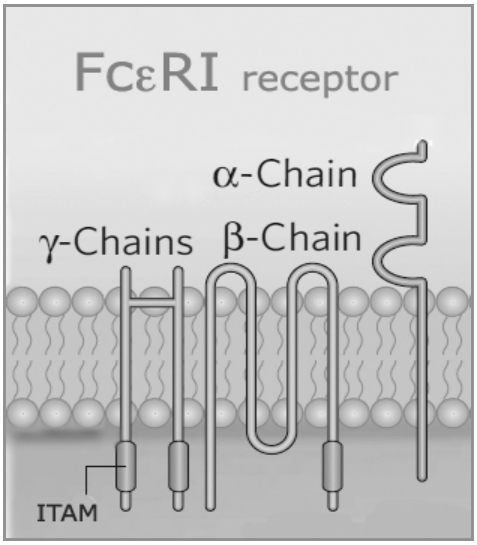


Figure 3. *High affinity receptor for IgE. An IgE-binding α chain, a membrane tetraspanning β chain with ITAMs and a disulfide-linked homodimer of γ-chains also with ITAMS.* ***Adapted from Galli SJ et al. (2)***

As a tetrameric molecule FcεRI is composed of three polypeptide subunits, an IgE-binding α chain, a membrane tetraspanning β chain and a disulfide-linked homodimer of γ-chains (***Figure 3***). This receptor lacks any intrinsic enzymatic activity but both β and γ chains contain immunoreceptor tyrosine-based activation motifs (ITAMs), which are essential for its signaling competence once phosphorylated.

The extracellular part of the α-subunit contains two extracellular immunoglobulin (Ig)-like domains (Metzger H / Garman SC et al.), which bind with a high affinity to the Fc part of monomeric IgE class antibodies. The γ-subunit is essential for the FcεRI-induced signal transduction and the β subunit has been proposed to function as an amplifier (Lin S et al.) and / or as a suppressor of the γ-chain-mediated signaling events (Furumoto Y et al.).

3.2 FcεRI signaling

The activation of mast cells, via its high affinity receptor FcεRI present on their surface, starts with the binding of the multimeric antigen to the receptor-bound IgE, thus initiating the cross-linking and aggregation of at least two receptors. Clustering of the receptors initiates multiple signaling pathways and internalization of the receptor complex and triggers a network of processes that are propagated inside the cell through a sophisticated network of signaling molecules that controls the cell response (Rivera J et al. / Gilfillan et al. (2)). A general outline of FcεRI signaling is represented in ***Figure 4***.

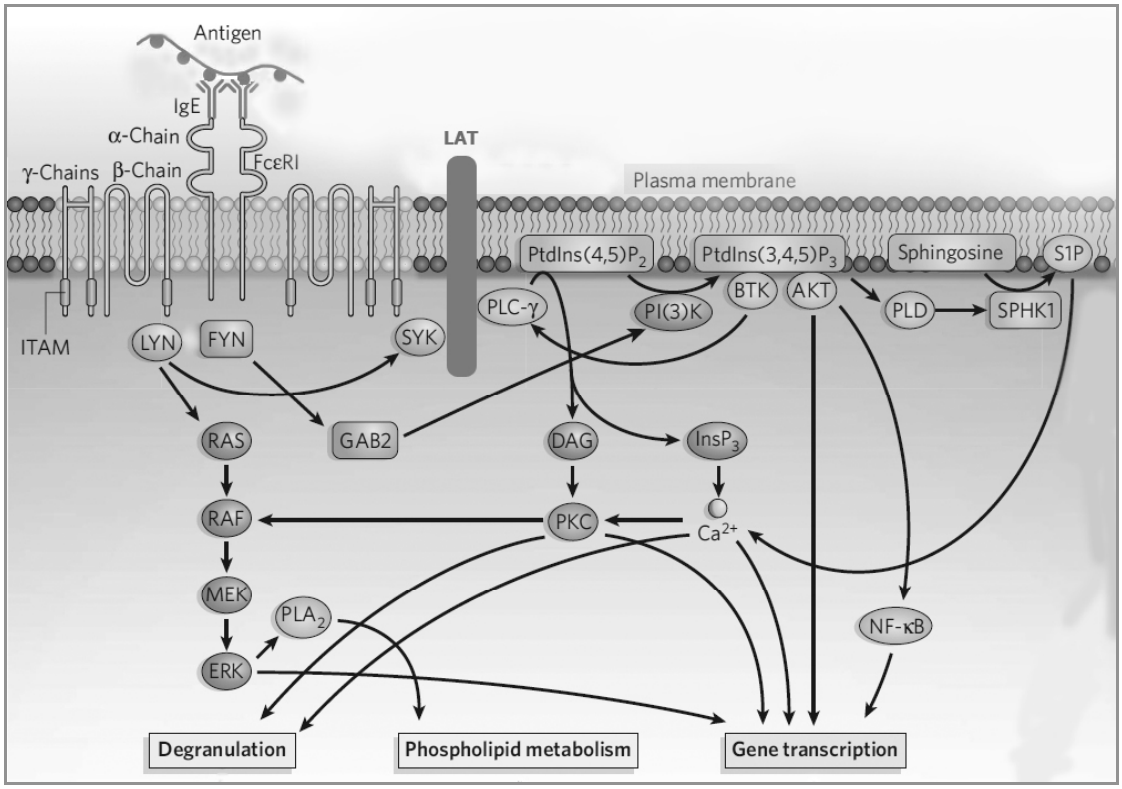


Figure 4. *Schematic representation of FcεRI signaling in mast cells.*
Adapted from Galli SJ et al. (2)

Since FcεRI lacks intrinsic tyrosine kinase activity, it associates with the nonreceptor Src family tyrosine kinase Lyn kinase, whose activity is key for phosphorylation of the tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAM) of the β and γ subunits of the receptor through transphosphorylation (Pribluda VS et al. / Jouvin MH et al.). Efficient phosphorylation of the receptor also requires plasma membrane liquid-ordered phase domains or lipid rafts that are enriched in cholesterol, sphingolipids and other saturated

phospholipids, as well as with a variety of signaling proteins including Lyn kinase (Young RM et al.). Coalescence of these dynamic cholesterol-rich domains (some of which might include Lyn) would stabilize or increase this transphosphorylation and cause assembly of a stable signaling complex.

Another Src family member, Fyn kinase, also appears to associate with the ITAM motif of FcεRIβ and, although it does not appear to participate in the phosphorylation of FcεRI, it is required for extracellular calcium entry, responsible for full degranulation and IL-2 cytokine production (Sanchez-Miranda E et al. / Rivera J et al.). Tyrosine phosphorylated receptor ITAMs recruit a variety of proteins that are key for signal amplification such as the protein spleen tyrosine kinase (syk) and tyrosine phosphatases SHP-1 and SHP-2.

Once activated, Fyn, Lyn, and Syk contribute to the formation of multi-molecular signaling complexes that are coordinated by adaptors, like LAT 1 and 2, Gab2, Grb2, Gads, among others (Alvarez-Errico D et al. / Saitoh S et al., (1) / Saitoh S et al. (2) / Kambayashi T et al. (2)). These signaling complexes provide docking sites for other signaling proteins including PLCγ1 and PLCγ2, SLP76, Vav1, Sos and others. PLCγ1 and PLCγ2 produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2). IP3 releases Ca2+ from intracellular stores as it binds to receptors in the endoplasmic reticulum (ER) and activates store-operated calcium channels (SOCs) for the influx of Ca2+ (Wang Z et al.). This increase in intracellular calcium concentration not only leads to PKC activation and degranulation but also plays a critical role in signals for de novo synthesis and secretion of eicosanoids and cytokines (Siraganian RP et al.), responsible for many signs and symptoms of allergic reactions.

4. Mast cell mediators

The result of mast cell activation is the appearance into the extracellular space of an impressive array of vasoactive mediators, proteases, chemokines, and cytokines that enhance vascular permeability, recruitment and function of leukocytes, and cause local inflammation (***Table 1***).

The granule-associated mediators, together with the newly synthesized lipid mediators, are responsible for many signs and symptoms of acute allergic reactions and anaphylaxis. In some cases, those mediators can recruit other inflammatory cells, and a late-phase reaction (sometimes more severe reaction) can develop a few hours later (Olivera A). Cytokines and chemokines can further amplify the allergic reaction by recruiting more inflammatory cells to the site.

<table>
<tr><th colspan="3">Mediators released by mouse mast cells</th></tr>
<tr><td>Biogenic Amines
Histamine
Serotonin</td><td>Proteases
mMCP-1, mMCP-2, mMCP-4,
mMCP-5, mMCP-6, mMCP-7
mMCP-9, mMCP-10 and mMCP-11
Carboxypeptidase A_3</td><td>Lysosomal enzymes
β-hexosaminidase
Cathepsins</td></tr>
<tr><td colspan="2">Proteoglycans
Heparin & chondroitin sulfate</td><td>Others
Peroxidase
MBP</td></tr>
<tr><td>Chemokines
CCL1
CCL2
CCL3,
CXCL1
CXCL2</td><td>Cytokines
TNF-α (preformed and newly generated)
IL-1, 3, 4, 5, 6, 9,10,13
MIP-1 family
TGF-β
IFN-γ</td><td>Lipid mediators
prostaglandins
cysteinyl leukotrienes
PAF</td></tr>
</table>

Table 1. *Mediators released by mouse mast cells*

Upon activation of mast cells, there are two possible outcomes: release of preformed mediators stored in the granules or de novo synthesis and secretion of mediators. The former occurs through a process known as “degranulation”. When mast cells degranulate, preformed compounds are released. Preformed mast cell mediators include well-known substances such as biogenic amines, lysosomal enzymes, proteoglycans, proteases and the preformed cytokine TNF-α (Wong GW et al. / Lundequist A. et al. / Humphries DE et al.). De novo synthesized mediators include lipid mediators and many cytokines and chemokines products of gene transcription and translation.

5. Tissue targets of mast cell mediators and related symptoms

Activated mast cells release large amounts of inflammatory mediators from their granules into the surrounding tissues. The effects of these mediators induce related organ symptoms (***Figure 5***). Reactions to drugs range from a mild localized rash to serious effects on vital systems. Severe allergic reactions affect primarily the skin, the upper and lower respiratory systems, the gastrointestinal system and the cardiovascular system.

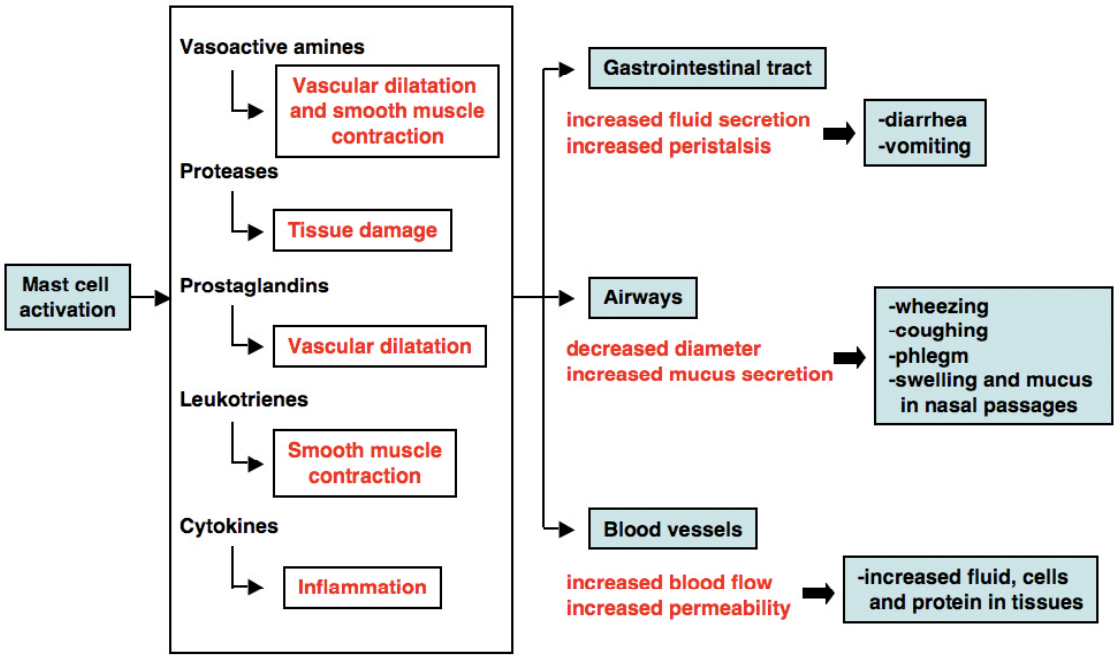


Figure 5. *Mediators released from mast cells and related organ symptoms*

Some systemic effects including vasodilation, mucous secretion, nerve stimulation, and smooth muscle contraction result in rhinorrhea, itchiness, dyspnea, laringeal edema, diarrhea, vomiting, hypotension and life-threatening circulatory collapse that can lead to death during anaphylaxis.

6. Hypersensitivity reactions involving mast cells

Multiple mechanisms of the innate and acquired immune system are required for defending the body against infections but immune responses are also capable of causing tissue injury and disease such as in hypersensitivity reactions. These reactions are classified following the principal immunologic mechanism responsible for tissue injury and disease such as:

- Immediate hypersensitivity (Type I), in which mast cells are involved
- Antibody-mediated (Type II)
- Immune complex-mediated (Type III)
- Tcell-mediated or delayed hypersensitivity (Type IV)

Immediate hypersensitivity (Type I), also called allergic reaction, is a rapid IgE antibody-and mast cell/basophil-mediated vascular and smooth muscle reaction, often followed by inflammation, that occurs in some individuals on encounter with certain foreign antigens to which they have been exposed and previously sensitized. Such reactions may affect various tissues and may be of varying severity in different individuals. Common types of immediate hypersensitivity reactions include hay fever, food allergies, bronchial asthma, and anaphylaxis.

Hypersensitivity reactions Type III are associated with IgG immune complexes and can be localized (Arthus reactions) or systemic (serum-sickness like reactions) or T cell-mediated (contact dermatitis).

We will focus in the immediate hypersensitivity Type I reactions.

6.1 Immediate hypersensitivity Type I: a two-step process

The development of an immediate hypersensitivity reaction, or allergic reaction, is a two-step process:

- Step 1 (***Figure 6***): Immediate hypersensitivity diseases are initiated by the introduction of an allergen, which stimulates Th2 reactions and IgE production. Dendritic cells internalize allergens and after processing them, display a recognizable portion of those molecules as an antigen to the naïve Tcells, which induce a Th2 response.

Activated Th2 cells secrete IL-4 and IL-13 cytokines and express CD40 ligand. B lymphocytes specific for an antigen express CD40 receptor. CD40 ligand-CD40 receptor signalling is critical for T cell dependent B cell activation, stimulating antigen specific B lymphocytes to switch to IgE-producing plasma cells (Elgueta R et al.). IgEs attach themselves to the surface FcεRI receptors of mast cells and remain on the lookout of specific antigens that triggers the mast cell response. Atopic individuals produce large amounts of IgE antibody in response to antigens that do not elicit IgE responses in the general population.

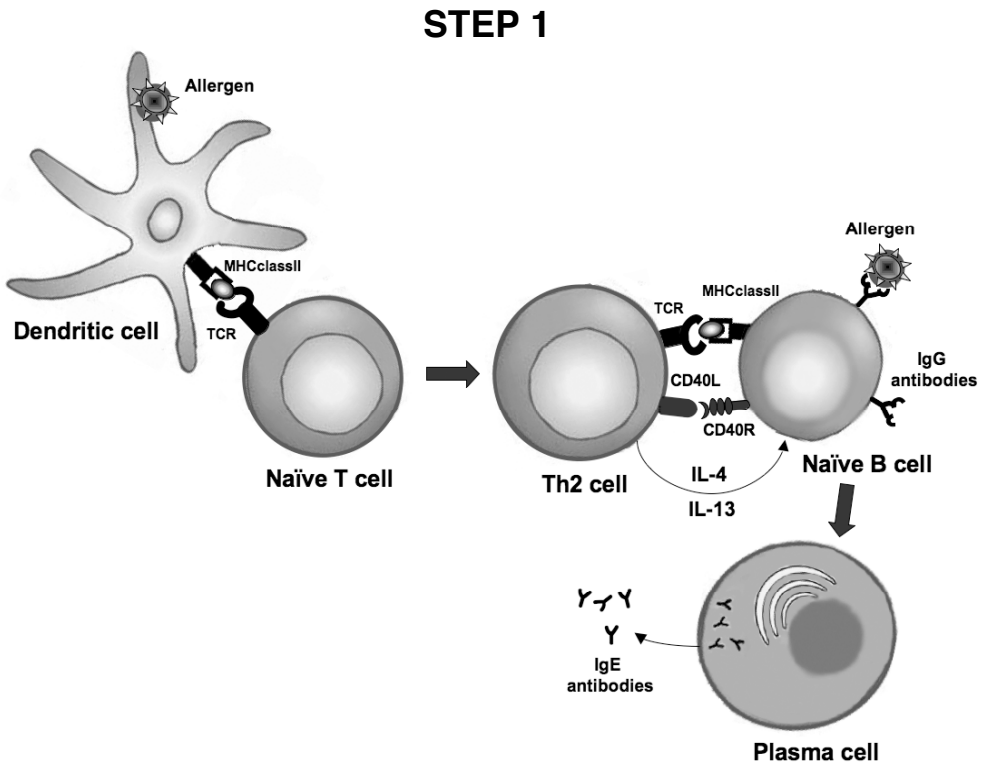


Figure 6. *Step 1 in an Immediate hypersensitivity Type I reaction. After a first allergen exposure, there is specific IgE antibodies production*

- Step 2 (***Figure 7***): The process of coating mast cells with IgE is called "sensitization". When mast cells sensitized by IgE are exposed to the allergen, the cells are activated by the binding of the allergen to two or more IgE antibodies on the surface of the cell (cross-linking of the bound IgE by the antigen), triggering biochemical signals leading to the release of mast cell mediators (Galli SJ et al. (2)).

Some mast cell mediators cause a rapid increase in vascular permeability and smooth muscle contraction that may occur within minutes, thus the name immediate hypersensitivity. Other mast cell mediators are cytokines that recruit neutrophils and eosinophils to the site of the reaction over several hours. This inflammatory component is called the late phase reaction, and it is mainly responsible for the tissue injury.

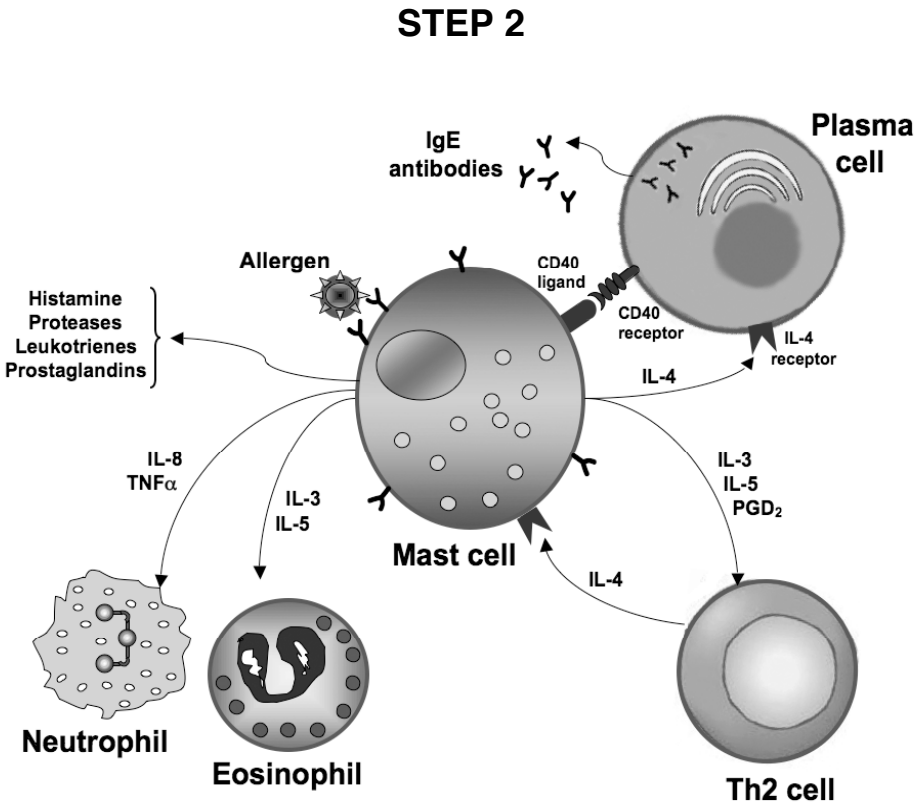


Figure 7. *Step 2 in an Immediate hypersensitivity Type I reaction. After a second allergen exposure, IgE antibodies recognize the allergen and trigger an allergic response*

6.2 Drug hypersensitivity: IgE and non-IgE mediated

Most people experience some type of adverse reaction to a medication at some point in their lives. Some of these adverse drugs reactions are mast cell-mediated hypersensitivity reactions, a subset of which occurs through an IgE-dependent mechanism.

Although any drug can potentially trigger an allergic response or some other type of hypersensitivity, some drugs have been recognized as more problematic than others (***Table 2***).

Drug-induced hypersensitivity reactions type I result from the release of mediators from IgE-sensitized mast cells or basophils and can affect all organ systems, leading to anaphylaxis and death. Cross-linking of IgE by drug antigens can lead to limited skin reactions or multiorgan system involvement, with decreased blood pressure and cardiovascular collapse during anaphylaxis. Reactions can occur within minutes of exposure and minimal amounts of the drug can induce severe reactions in highly sensitized individuals, such as laryngeal edema with asphyxiation.

Drug induced hypersensitivity reactions		
Antibiotics • cephalosporins • penicillin • sulfonamides • vancomycin	**Monoclonal Antibodies** • anti-TNF-α (Infliximab) • anti-CD-20 (Rituximab) • anti-CD-340 (Trastuzumab)	**Aspirin and nonsteroidal anti-inflamatory drugs** • acetylsalicylic acid • ibuprofen • naproxen
Insulin • porcine insuline • recombinant human insulin	**Chemotherapeutic agents** • platins (carboplatin, oxiplatin, cisplatin) • taxanes (paclitaxel and docetaxel)	**Others** • anticonvulsants • anesthesic agents • blood products • morphine derivatives • antiretrovirals

Table 2. *Some of the drug antigens that can induce hypersensitivity reactions.*

Drug antigens can sensitize patients after multiple courses, and repeated exposures are needed for the development of specific IgE. Sensitizing drugs can act as complete antigens, such as insulin, or haptens, which are coupled to a carrier protein, such as penicillin. Among chemotherapy drugs, patients sensitive to carboplatin typically presented on their seventh to tenth drug exposure with predominantly cutaneous, cardiovascular, respiratory, and gastrointestinal symptoms, a pattern consistent with anaphylaxis. These reactions are caused by the rapid release of preformed and newly synthesized mediators from sensitized mast cells through the cross-linking of FcεRI by drug antigens. Patients reacting to paclitaxel, however, experienced chest pain, back pain, oxygen desaturation, hypertension, and presyncope on their first or second exposure, which are symptoms presumed to be due to IgE-independent mechanisms (Castells MC et al. (3) / Sheffer AL et al.).

7. Mast cell rapid IgE desensitization

Drug hypersensitivity reactions can occur with most drugs. In most cases, the suspected drug is avoided in the future. However, first line therapy may prolongue the patient's life in the case of cancer patients or provide a better quality life for patients with chronic inflammatory conditions. Clinicians must decide which agent is the best for a particular patient with a given disease. Adverse drug reactions are frequently encountered and threaten to relegate patients to a secondary therapy.

Adverse drug reactions inducing a type I hypersensitivity reaction, whether IgE or non-IgE mediated, are eligible for rapid desensitizations. The symptoms of these reactions include cutaneous, respiratory, gastrointestinal, cardiovascular, neuromuscular, and/or throat tightness during the infusion or shortly after the administration of these medications. Under these circumstances, desensitization may be performed to induce a temporary state of tolerance to a drug, allowing patients with such a drug hypersensitivity to receive the optimal agent for their disease.

Rapid desensitization is an effective technique for safely administering important medications while minimizing or entirely circumventing such adverse reactions in sensitized patients.

7.1 Protocols for human rapid desensitizations

Rapid desensitization protocols have been developed and are used in patients with allergic reactions to antibiotics (mainly penicillin), insulins, sulfonamides, chemotherapeutic and biologic agents, and many other drugs.

Desensitization protocols are performed by administering increasing doses of the medication concerned over a short period of time (from several hours to a few days) until the total cumulative therapeutic dose is achieved and tolerated. It is a high-risk procedure used only in patients in whom alternatives are less effective or not available after a positive risk/benefit analysis.

The Brigham and Women's Hospital (BWH) Desensitization Program, under Dr. Castells direction, devised a 12-step standard protocol that is safe and effective and has been used for desensitization to three different drugs: chemotherapic agents (carboplatin), monoclonals (infliximab) and antibiotics (Ceftazidime). An example of each protocol is shown in ***Figure 8***.

Rapid Drug Desensitization protocols

A. **Carboplatin** (Chemotherapic agent)

Step	Solution	Rate (ml/h)	Time (min)	Administered dose (mg)	Cumulative dose (mg)
1	A	2	15	0.025	0.025
2	A	5	15	0.063	0.088
3	A	10	15	0.125	0.213
4	A	20	15	0.250	0.463
5	B	5	15	0.625	1.088
6	B	10	15	1.250	2.338
7	B	20	15	2.500	4.838
8	B	40	15	5.000	9.838
9	C	10	15	12.500	22.338
10	C	20	15	25.000	47.338
11	C	40	15	50.000	97.338
12	C	75	64.4	402.663	500.000
		Total time = 3.8 h		Total dose = 500 mg	

B. **Infliximab** (Monoclonal)

Step	Solution	Rate (mL/h)	Time (min)	Volume infused per step (mL)	Dose administered with this step (mg)	Cumulative dose (mg)
1	1	2.0	15	0.50	0.012	0.012
2	1	5.0	15	1.25	0.030	0.042
3	1	10.0	15	2.50	0.060	0.102
4	1	20.0	15	5.00	0.120	0.222
5	2	5.0	15	1.25	0.300	0.522
6	2	10.0	15	2.50	0.600	1.122
7	2	20.0	15	5.00	1.200	2.322
8	2	40.0	15	10.00	2.400	4.722
9	3	10.0	15	2.50	5.953	10.675
10	3	20.0	15	5.00	11.906	22.580
11	3	40.0	15	10.00	23.811	46.392
12	3	80.0	174.38	232.50	553.609	600.000
Total time = 340 min (5.66 h)						

B. **Ceftazidime** (Antibiotic)

Step	Solution	Rate (mL/h)	Time (min)	Volume infused per step (mL)	Dose administered with this step (mg)	Cumulative dose (mg)
1	1	2.0	15	0.50	0.100	0.100
2	1	5.0	15	1.25	0.250	0.350
3	1	10.0	15	2.50	0.500	0.850
4	1	20.0	15	5.00	1.000	1.850
5	2	5.0	15	1.25	2.500	4.350
6	2	10.0	15	2.50	5.000	9.350
7	2	20.0	15	5.00	10.000	19.350
8	2	40.0	15	10.00	20.000	39.350
9	3	10.0	15	2.50	49.016	88.366
10	3	20.0	15	5.00	98.0325	186.399
11	3	40.0	15	10.00	196.065	382.464
12	3	80.0	61.87	82.50	1617.536	2000.00
Total time = 226.875 minutes (3h 46m)						

Figure 8. A. *All reported results have been generated at the Desensitization Unit of the Brigham and Women's Hospital. Desensitization protocol for a total carboplatin dose of 500 mg* ***Adapted from Lee CW et al. (2) B.*** *Desensitization protocol for intravenous infliximab (600 mg).* ***Adapted from Brennan PJ et al. C.*** *Desensitization protocol for a total ceftazidime dose of 2 g*, using solution volumes of 100mL. ***Adapted from Legere HJ 3rd et al.***

The most commonly used protocol has 12 steps, using three 10-fold diluted solution bags, at escalating rates. Patients who have had severe anaphylactic reactions to the agent of choice or who have reacted early in the standard 12-step desensitization may experience fewer symptoms if desensitized using a 16-step protocol, which adds another bag containing 1/1000th of the full dose. The use of a 16-step (four solution bags) or a 20-step (five solution bags) protocol is reserved for high-risk patients. Common side effects include flushing, warmth, pruritus, erythema, and urticaria, and patients are cautioned about the low but real risk of anaphylaxis. No life-threatening HSRs or deaths occurred during the procedure, and all patients received their full target dose. Most reactions occurred during the first desensitization. Reactions were most commonly reported at the last step of the protocol (***Figure 9***).

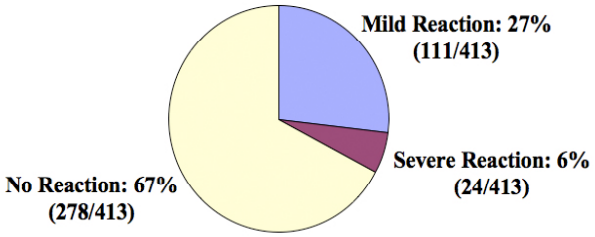
The BWH standardized desensitization protocol is a dynamic and a flexible protocol that begins with an analysis of the patient's hypersensitivity reaction, design and testing of an initial desensitization protocol, and adjustment of this protocol in an iterative fashion based on the patient's response.

The steps followed are:

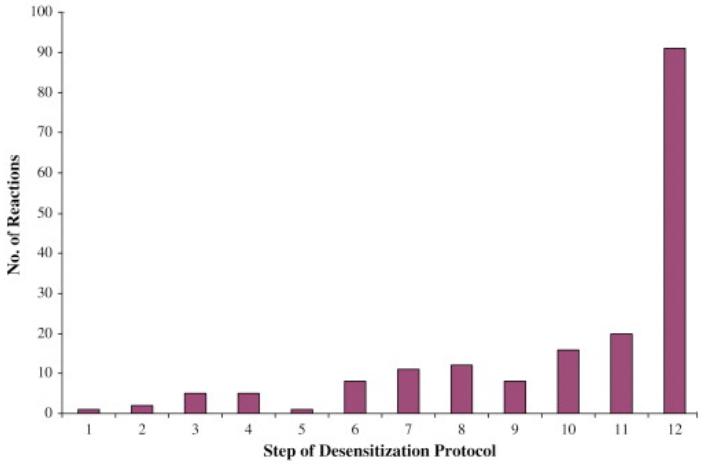
1. Evaluate the patient, attempting to characterize the nature of a patient's adverse reaction.
2. Determine the likelihood that rapid drug desensitization will be effective and safe.
3. Apply or design a reasonable desensitization protocol (often using our standard 12-step protocol as a starting place)
4. Collect information about how the patient responds to each desensitization and modify the protocol as needed:
   (a) adding, subtracting or changing premedications
   (b) changing the number of steps in the protocol
   (c) altering the rate or time of one or more steps
   (d) some combination of these

Over the past 10 years, more than 99.9% of nearly 800 patients have received the full dose of their first-line medication in thousands of desensitizations at BWH, and there have been no deaths from hypersensitivity reactions. Rapid drug desensitization protocols have been remarkably successful and hundreds of patients with infections, cancer, and inflammatory conditions have been treated, providing improved quality of life and increased survival rates. However, the molecular basis of rapid desensitization is not completely understood and in-vitro studies are needed to do so. (Liu et al.)

A. Number and severity of reactions during desensitization



B. Desensitization step at which reactions occurred



C. Desensitization course at which reactions recurred

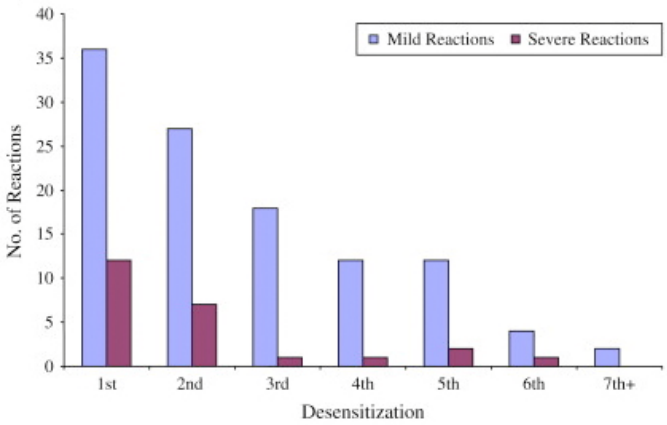


Figure 9. A. *Number and severity of reactions during the desensitization process. A mild reaction was defined as absence of chest pain, changes in blood pressure, dyspnea, oxygen, desaturation, or throat tightness. A severe reaction included 1 of these.* ***B.*** *Desensitization step at which reactions occurred (total number of reactions 5 180).* ***C.*** *Desensitization course at which reactions recurred. Total number of reactions 135 (111 mild and 24 severe).* ***Adapted from Castells MC et al. (3)***

7.2 Protocols for in-vitro rapid desensitizations

Since all clinical desensitization protocols are empiric and based on error and trial clinical experiences, basic research have been done to uncover the cellular and molecular mechanisms underlying the temporary toleration induced by rapid desensitization, thus improving its safety and efficacy (Castells MC et al. (3)).

Mast cells and basophils seem to be targets in the process since mediators from these cells are released during hypersensitivity reactions to drugs, as well as during desensitization procedures. After rapid desensitization, specific skin test reactivity is abolished, indicating that the allergen is no longer able to trigger skin mast cell activation and that systemically distributed mast cells have lost the ability to release mediators (Lee CW et al. (1)).

In vitro desensitization protocols performed with mast cells and basophils have been developed to unrable the mechanisms underlying successful in vivo desensitizations.

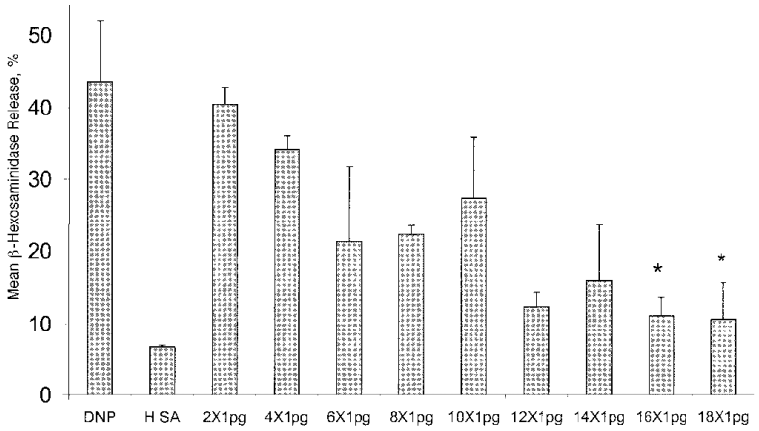
First *in vitro* desensitization data were reported in 1979 by Lichtenstein's group using human basophils passively sensitized with serum from penicillin and ragweed allergic patients (Sobotka AK et al. / Dembo M et al.). Later on, *in vitro* desensitizations of human basophils were done in the presence of calcium, with increasing doses of IgG anti-IgE (Mendoza GR et al.) or incubating them with suboptimal concentrations of antigen for 45 minutes (Pruzansky JJ et al.). More recently, prolonged antigen incubation has been used to desensitize human mast cells and basophls (MacGlashan DJr (5) / Macglashan D et al. (2) / Kepley CL (2)/ Komiya A et al.).

Same kind of *in vitro* desensitization approaches were done to render mast cells unresponsive to antigens, by exposure to low antigen doses in calcium depleted conditions (Ishizaka T et al.) or, in the presence of calcium, using repeated doses of antigen (Rubinchik E et al.) or incremental doses of antigen with long time periods between steps (Shalit M et al. / Rubinchik E et al.).

The very first publication from Dr. Castells' group related to mouse mast cell rapid desensitization was done in 2005 (Morales AR et al.). In this study, two different antigens (DNP-HSA or TNP-HSA) were used for the evaluation of activation and rapid desensitization of mouse BMMCs. Two protocols were developed, a first one, using repetitive suboptimal doses of antigen (18 doses of 1pg) at 5 min intervals and a second one, using doubling doses of antigen (1, 2, 4, 8, 16, 32, 64, 128, 256 and 500 pg) at 10 min intervals.

They showed (***Figure10***) that rapid desensitization was dose dependent (cells were successfully desensitized with 16 and 18 doses of 1 pg DNP-HSA at 5-minute intervals) and time dependent (delivering doses too fast delayed unresponsiveness).

A. Dose dependency



B. Time dependency

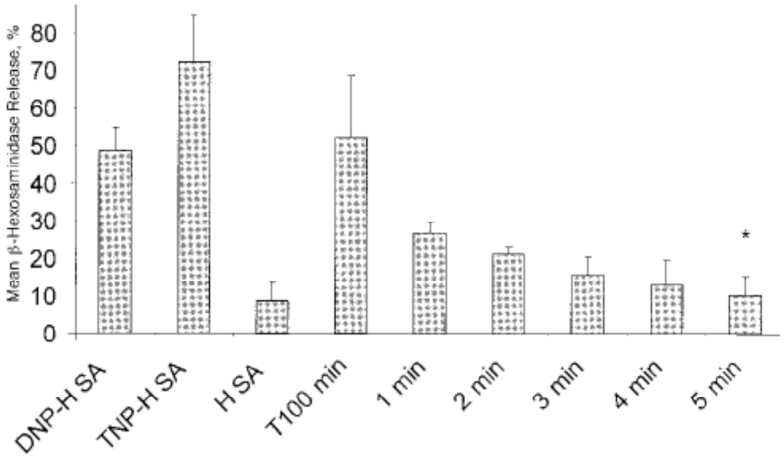


Figure 10. A. *β-hexosaminidase release obtained with rapid desensitization of mBMMCs by several number of single repetitive 1-pg doses of dinitrophenyl (DNP) human serum albumin (HSA).* ***B. β-****hexosaminidase release obtained when DNP-HSA, TNPHSA, or HSA (1 ng) was added to mBMMCs sensitized with DNP-IgE or TNP-IgE. For desensitization, doses were delivered at 1-, 2-, 3-, 4-, and 5-minute intervals (columns 5–9).* ***Adapted from Morales AR et al.***

Furthermore, they showed that mBMMCs from BALB/c STAT6-null mice had no statistically significant difference with activated cells, indicating a failure of desensitization, thus pointing the transcription factor STAT6 as a molecular target for desensitization (***Figure 11***).

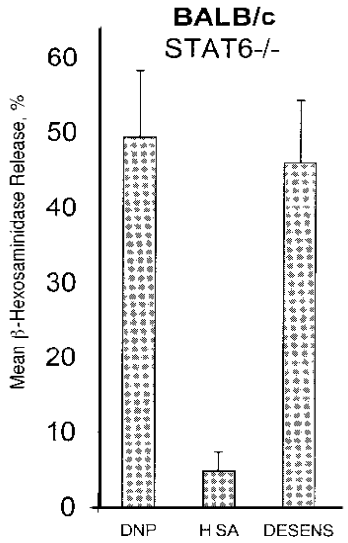


Figure 11. *Signal transducer and activator of transcription 6 (STAT6)–null mouse BMMCs from BALB/c cannot be rapidly desensitized to dinitrophenyl (DNP) human serum albumin (HSA). The mBMMCs from BALBc STAT6-null mice were activated with DNP-HSA (column 1), HSA (column 2), or desensitized (DESENS) (column 3)* ***Adapted from Morales AR et al.***

Five hypotheses (***Figure 12***) explaining how rapid desensitization could impair mast cell activation have been articulated:

1. Depletion of activating signal transduction components such as syk kinase (Gilfillan AM et al. (1) / Kepley CL (1) / Gomez G et al. / Kepley CL et al. (2))
2. Blockade of the components for signal transduction, with low Ag dosis or monomeric antigens, that avoid the aggregation and the crosslinking of the FceRI receptors engaged to the IgE (Paolini R et al.)
3. Sub-threshold depletion of mediators (Shalit M et al. / Rubinchik E et al.)
4. Internalization of FcεRI through progressive cross-linking at a low antigen concentration (Shalit M et al.)
5. Activation of inhinitory receptors capable of bloking the signal transduction for mast cell activation (Castells MC et al. (2))

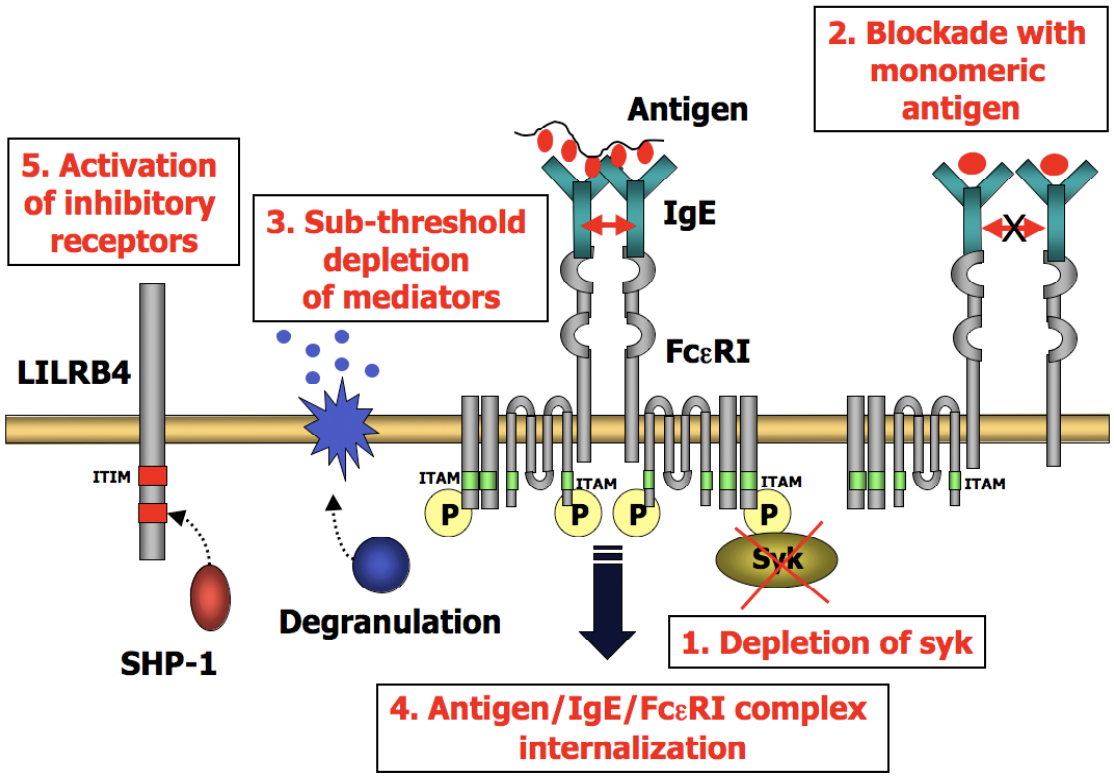


Figure 12. *Schematic representation of five suggested hypotheses of mast cell desensitization.*

The present PhD thesis describes an improved physiologic model of rapid mouse BMMCs desensitization to IgE antigens, studies its kinetics, reproducibility, effectiveness and versatility and provides the first mechanistic basis for the profound mast cell inhibition resulting from the process.

OBJECTIVES

Our main goal is to generate a reproducible, effective and versatile *in vitro* rapid desensitization protocol for murine bone marrow derived mast cells (mBMMC), under physiological calcium conditions, and elucidate:

- the kinetics, the duration and the specificity of the desensitization
- the molecules involved in the signal transduction with desensitization
- the acute and late phase mast cell responses with desensitization
- the fate of the FcεRI/IgE/antigen complex with desensitization

MATERIAL & METHODS

CHAPTER 1: General techniques

1. Cell cultures

1.1 Bone Marrow Mast Cells

BMMCs obtention

Male BALB/c mice 8–12 wk old (Jackson Laboratory) were euthanized by CO_2 asphyxiation. Mouse fur was wet with 70% ethanol and the skin was cut off legs. Foot was removed by cutting below the ankle joint and the leg was cut off above hip. Under a biological hood, the muscle was trimmed away and the bone was cut off at the ends. Then, 3 ml of culture medium was passeed through each end of the bone using a 3-cc syringe with a 30-G needle. The medium was collected in a 50-ml conical centrifuge tube. Cells were resuspended in 10 ml of BMMC medium with IL-3 and transfered to a 75-cm^2 tissue culture flask already with 10 ml. Flasks was incubated upright at a 37°C in a 5% CO_2 incubator. Cells were maintained at $1x10^6$ cells/ml passing them every 5-6 days. Before BMMCs were used for assays, cells were cultured at least for 4-5 weeks or until c-Kit FACS is positive.

Culture medium for BMMCs: 500 ml RPMI 1640 medium without glutamine (GIBCO) supplemented with 6 ml 100Å~ Penicillin-Streptomycin-Glutamine (Invitrogen), 12.5 ml 1 M HEPES (Invitrogen), 5 ml 100Å~ nonessential amino acids (Sigma-Aldrich), 60 ml fetal bovine serum (FBS) and 10 ng/ml IL-3 (obtained from 293T as described below).

1.2 293-T cell line

Thaw 293-T cells and place in DMEM Media. Pass cells first time at 24h with 25 ml DMEM media in 175 cm^2 flasks.

Culture medium for 293-T: 500 ml Earle's Minimal Essential Medium (Cellgro) supplemented with 100 ml fetal bovine serum (FBS) and 6 ml 100Å~ Penicillin-Streptomycin-Glutamine (Invitrogen).

1.3 RBL-2H3 cell line

Thaw RBL-2H3 cells and place in DMEM Media. Pass cells first time at 24h, with 25 ml DMEM media in 175 cm^2 flasks.

Culture medium for RBL-2H3: 500 ml Earle's Minimal Essential Medium (Cellgro) supplemented with 100 ml fetal bovine serum (FBS) and 6 ml 100Å~ Penicillin-Streptomycin-Glutamine (Invitrogen).

2. Recombinant IL-3 production

DNA production

E.coli transformed with mouse IL-3 vector (Yokota T et al. / Niwa H et al.) in LB (Luria Bertani) media with 1μg/mL of Ampicilin (Sigma) in a shaker overnight at 37°C. Bacteria was collected by centrifugation at 6000rpm 4ºC for 15 minutes and pellets were kept at -20ºC. Plasmid was purified with a Quiafilter Plasmid Midi Kit (Quiagen) following manufacturer protocol. DNA was measured by optical density and adjusted to 1μg/μL. Vector was kept at -80°C.

DNA transfection

293-T cells at 70% of confluence in 5 plates (13 mm Molded-In Grid, BDBiosciences) were transfected with 22 μg of mouse IL-3 vector with Fugene following manufacturer protocol. Supernatants were collected 48 h. later, mixed and filtered in a 0.2 μm Millipore, aliquoted in 10 ml tubes and kept at -70º C. IL-3 concentration was determined with Peprotech kit for IL-3 determination.

3. OVA IgE production

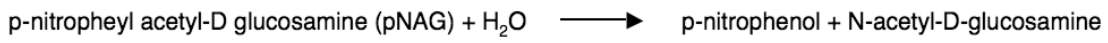
BALB/c mice were injected via i.p. with 200 μL/mouse of *sensitization solution* on days 1,7 and 14. 30 days later, challenged mice with 200 μL/mouse of OVA shot. After 5 days blood were harvested from anesthetized mice via cardiac puncture. Blood were placed in eppendorfs at RT[a] for 30 min, centrifuged at 10,000 rpm for 15 min. Serum were aliquoted and kept at -80°C.

Sensitization solution (1.5 mL):
- OVA solution: 50 mg OVA powder were diluted in 50 mL with HBSS and filtered
- Sensitization solution: 750 μL OVA solution were mixed with 750 μL Alum suspension in a 50 mL tub. Tube was shaked more than 2 hours.
- OVA shot (2mL): 50 mg OVA powder diluted in 50 mL HBSS- - and filtered

4. β-hexosaminidase release assay

β-hexosaminidase is a lysosomal enzyme that hydrolyzes terminal nonreducing N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides (Aronson NN Jr et al.). It acts on glucosides, galactosides, and several oligosaccharides. Mast cell granules contain large quantities of this enzyme due to the lysosomal nature of their granules, which are also called secretory lysosomes.

The assay exploits the hydrolytic property of β-hexosaminidase and measures catalysis of the following reaction:



The amount of p-nitrophenol formed is directly proportional to the amount of β-hexosaminidase in the supernatant or in the cell lysates and absorbs light at 410 nm, thus providing a good quantitative assay for measuring mast cell degranulation (Schwartz LB et al. (1) / Schwartz LB et al. (2)).

4.1 Protocol for mBMMCs

After activation, control or desensitization, cells were centrifuged to separate supernatants and were resuspended in 100 μL of fresh media each. Cells were disrupted by three 5-minute cycles of freeze (dry ice slurry) and thaw (37°C bath), and cellular debris was removed by centrifugation. 20 μL from each supernatant and pellet in duplicate were added to 80 μL of *β-hexosaminidase cocktail* in a 96-well microtiter plate (F-Immuno Module; Costar Corning Inc, Corning, NY). Medium alone was added as control. After incubation at 37°C for 30 minutes in the dark, 200 μL of *stop solution* was added, and fluorescence was read at 405 nm on a kinetic microplat enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA).

$$\% \beta\text{-hexosaminidase release} = \frac{\text{release in supernatant}}{\text{release in supernatant + release in pellet}} \times 100$$

β-hexosaminidase cocktail for BMMCs (pNAG substrate):
70 mL of 0.2M Na_2HPO_4 were mixed with 20 mL of 0.4 M citric acid monohydrated and pH adjusted to 4.5. The former solution was used to prepare 4 mM *p*-nitrophenyl acetyl-D-glucosamine (Sigma), vortexing and warming to 37°C to dissolve. Aliquots were stored at –20°C.

Stop solution for BMMCs:
0.2 M glycine solution and adjust pH to 10.7.

4.2 Protocol for RBL-2H3 cell line

Cell supernatants and cell lysates from desensitization, activation, control or spontaneous release were used for β-hexosaminidase release assay. 25 μL of each supernatant and cell lysate in duplicate were added to 50 μL of *β-hexosaminidase cocktail* in a well of a flat-bottom 96-well plate. After incubation at 37°C for 90 minutes in the dark, 150 μL of *stop solution* was added, and absorbance was read at 405 nm on a kinetic microplat enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA).

$$\% \text{ net specific release} = \frac{\text{release stimulated - release spontaneous}}{\text{total content - release spontaneous}} \times 100$$

β-hexosaminidase cocktail for RBLs (pNAG substrate)**:** 1 mM *p*-nitrophenyl acetyl-D-glucosamine (Sigma) was prepared in *citrate buffer* by vortexing and warming to 37°C to dissolve. Aliquots were stored at **–**20°C.

Citrate buffer 0.1 M, pH 4.5*:* 26.5 ml of 0.1 M citric acid were mixed with 23.25 ml of 0.1 M sodium citrate. The solution was filtered to sterilize.

Stop solution for RBLs (0.1M Carbonate buffer)**:** 1.06 g Na_2CO_3 and 0.840 g $NaHCO_3$ were dissolved in 100 ml of distilled H_2O, filtered and pH adjusted to 9.

Tyrode's buffer for all RBL experiments: 135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl2, 1 mM MgCl2, 0.5 mg/ml BSA, 20 mM HEPES. Adjust pH to 7.3 with NaOH.

5. Measurement of calcium flux

Desensitized, non-desensitized and non-IgE treated cells were washed and resuspended in HBSS containing 1mM CaCl2, 1mM MgCl2 and 0.1% BSA (Buffer A). Cells were then loaded with 2.5 **m**M Fura-2AM (from Molecular Probes) in the presence of 2.5mM probenecid for 30 min at 37°C following a published protocol (Maekawa A et al.). After being labeled, cells were washed and resuspended in cold Buffer A (0.5 $x10^6$ cells/mL). Fluorescence output was measured with excitation at 340 and 380nm in the F-4500 Fluorescence Spectrophotometer (Hitachi), and the relative ratio **(R)** of fluorescence emitted at 510nm was recorded. For all fluorescence ratios to start at zero, the first fluorescence value of each sample was subtracted from all its subsequent fluorescence values.

6. ELISAs: IL-3, IL-6 and TNF-α

IL-3

Supernatants containing IL-3 obtained from 293T transfected cells were analized according to manufacturer's protocol (Peprotech).

IL-6 and TNF-α

After desensitization or challenge, TNF-α and IL-6 contents in cell-free supernatants were estimated using a mouse TNF-α or IL-6 ELISA kits (eBioscience), either 30 min or 4 h after activation or desensitization, according to the manufacturer's protocol.

7. Immunoblot analysis

After desensitization or challenge, cells were collected and washed with cold PBS. Pellets were lysed in *RIPA buffer* supplemented with protease and phosphatase inhibitor cocktails (Roche). Total protein lysates were subjected to SDS-PAGE on a 4–12% polyacrylamide gel and transferred to a nitrocellulose membrane (both from Invitrogen). Membranes were blotted with anti-Phospho-STAT6 (phosphotyrosine 641) and anti-STAT6 from Sigma-Aldrich or with anti-LAT and anti-Phospho-LAT (Tyr 191) or with anti-Phospho-p38MAP kinase and anti-p38MAP kinase from Cell Signaling. Signal detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RIPA buffer:
Tris-HCl 50mM, pH 7.4
NP-40 1%
Na-deoxycholate 0.25%
NaCL 150mM
EDTA 1mM
NaF 1mM
PMSF 1mM
Aprotitin, Leupeptin, Pepstatin: 1ug/ml each
Na3VO4 1mM

RIPA buffer was kept at -20°C until ready to use. Protease, phosphatase inhibitors (Roche) and 100μl of 100mM PMSF and 100μl of Na3VO4 100mM (per 10ml of lysis buffer) were added to the solution thesame day the assay was run.

8. Flow cytometry analysis

After desensitization or challenge, cells were placed at 4°C, then washed and resuspended in PBS containing 0.5% BSA and 0.05% sodium azide at 4°C and incubated with anti-FcγRI/II mAb (eBioscience) for 20 min on ice to block Fcγ receptors. Cells were then incubated with 5 mg/mL of FITC rat anti-mouse IgE (BD Biosciences) or 2 mg/mL of PE Armenian hamster anti-mouse FcεRIα (eBioscience) or with the recommended isotype controls. Cells were analyzed on a BD Biosciences FACS Canto flow cytometer, using FACSDiva acquisition software and FlowJo analysis software.

9. RP-HPLC analysis

After desensitization or challenge, cell supernatants were collected and LTB4, LTC4 and 12-HHT were measured by RP-HPLC following a published protocol (Hsieh FH et al.). Briefly, samples were applied to a C18 Ultrasphere RP column (Beckman Instruments) equilibrated with a solvent consisting of methanol/ACN/water/acetic acid (10:15:100:0.2, v/v), pH 6.0 (Solvent A). After injection of the sample, the column was eluted at a flow rate of 1 mL/min with a programmed concave gradient to 55% of the equilibrated Solvent A and 45% of Solvent B (100% methanol) over 2.5 min. After 5 min, Solvent B was increased linearly to 75% over 15 min and maintained at this level for an additional 15 min. The UV absorbance at 280 and 235nm and the UV spectra were recorded simultaneously. PGB2 was used as an internal standard.

10. Confocal microscopy

Antigens used were Alexa Fluor 488-conjugated OVA (from Molecular Probes) and DyLight Fluor 649-conjugated DNP, labeled with DyLight 649 NHS Ester (Thermo Scientific). Due to detection limitations, OVA activation dose was 50 ng, DNP activation dose was 5 ng and the rapid OVA desensitization protocol was consequently adjusted based on the volumes used in the protocol in Table 1 but at higher concentrations. After desensitization or challenge, cells were washed and resuspended in cold PBS. Cells were transferred onto poly-L-lysine-coated round cover slips for 20 min at 4°C and then fixed with 4% paraformaldehyde in PBS for 10 min at 4°C. After three washes with PBS, cells were incubated with cholera toxin subunit B-Alexa Fluor 555 conjugate (Molecular Probes) 1:500 in PBS for 10 min at 4°C, washed three times with PBS and mounted using an aqueous mounting medium (15% wt/v polyvinyl alcohol, 33% v/v glycerol, 0.1% azide). Images were collected sequentially using a 63x plan Apo NA 1.4 objective on Leica SP5X laser scanning confocal system attached to an inverted Leica DMI6000 microscope.

CHAPTER 2: In-vitro protocol for rapid desensitization

1. Design of the rapid desensitization protocol for BMMCs to DNP and OVA antigens

The initial human protocols for penicillin desensitization that successfully reintroduced penicillin in allergic patients started with 1/100 to 1/1,000 the target dose, and doubling or 10-fold increments of that dose were administered at fixed intervals until the target dose was reached (Wendel G et al. / Sullivan T et al.). More recent protocols (Lee CW et al. Gynecol Oncol. (1) / Castells MC et al. (3) / Legere HJ 3rd / Brennan PJ et al.) have provided support for these early empirical starting doses and for the need for interval times between doses.

Based on human data and on previous data of Dr. Castells' group (Morales AR et al.), an *in vitro* desensitization protocol using BALBc mouse BMMCs was generated. In our protocol, eleven doses of antigen DNP-HSA are delivered to mouse BMMC at fixed 10-minutes-time intervals until the target dose, 1 ng of DNP-HSA, is reached. The antigen solutions used are of the following concentrations: 1 pg/μL, 5 pg/μL, 10 pg/μL and 20 pg/μL.
As described in ***Table 3***, the first dose is a non-triggering dose of 1 pg in the DNP system and 10 pg in the OVA system which is 1/1000 of the target dose.

Steps	Time (min)	Volume (μL) DNP-HSA	Concentration (pg/μL) DNP-HSA	Dose (pg) DNP-HSA
1	0	1	1	1
2	10	1	5	5
3	20	1	5	5
4	30	1	10	10
5	40	1	10	10
6	50	2	10	20
7	60	2	20	40
8	70	4	20	80
9	80	8	20	160
10	90	16	20	320
11	100	17.5	20	350
11 steps	100 min	54.5 μL*		1 ng

*added to 50 μL of cells

Table 3. *Rapid desensitization protocol in which eleven incremental doses of antigen DNP-HSA were delivered to mBMMCs at fixed time intervals until the target dose (1 ng DNP) was reached.*

In the four following steps doses are doubled and repeated (5, 5, 10 and 10 pg) and from the 5th dose on, doses are doubled (20, 40, 80, 160 and 320 pg). The last dose is 350 pg which is the amount of picograms left in order to accomplish the target dose (1 ng DNP-HSA).

In our protocol for OVA rapid desensitization, eleven doses of antigen OVA are delivered to mouse BMMC at fixed 10-minutes-time intervals until the target dose, 10 ng of OVA, is reached. The antigen solutions used are of the following concentrations: 10 pg/μL, 50 pg/μL, 100 pg/μL and 200 pg/μL.

As described in ***Table 4***, the first dose is 10 pg which is 1/1000 of the target dose. In the four following steps doses are doubled and repeated (50, 50, 100 and 100 pg) and from the 5th dose on, doses are doubled (200, 400, 800, 1600 and 3200 pg). The last dose is 3500 pg which is the amount of picograms left in order to accomplish the target dose (10 ng OVA).

Steps	Time (min)	Volume (μl) OVA	Concentration (pg/μl) OVA	Dose (pg) OVA
1	0	1	10	10
2	10	1	50	50
3	20	1	50	50
4	30	1	100	100
5	40	1	100	100
6	50	2	100	200
7	60	2	200	400
8	70	4	200	800
9	80	8	200	1600
10	90	16	200	3200
11	100	17.5	200	3500
11 steps	100 min	54.5 μl*		10 ng

*added to 50 μL of cells

Table 4. *Rapid desensitization protocol in which eleven incremental doses of antigen OVA were delivered to mBMMCs at fixed time intervals until the target dose (10 ng OVA) was reached.*

The release of the lysosomal enzyme β-hexosaminidase after stimulation correlates well with the release of histamine and it is used as a measure of mast cell degranulation. β-hexosaminidase assay is well described in CHAPTER 1, section 4.

2. Activation and rapid desensitization of BMMCs to DNP and OVA

DNP antigen: Cells were sensitized overnight with anti-DNP IgE (0.25 μg/10^6 cells/mL). The next day, cells were washed to eliminate possible excess of unbound IgE, resuspended in 50 μL of fresh medium without IL-3 and placed at 37°C. For desensitization, cells were treated as per Table 1 (rapid desensitization protocol), and 10 min after the last DNP-HSA addition, placed on ice for β-hexosaminidase release assay. For activation, cells were challenged with 50 μL of DNP-HSA at 20 pg/μL (1 ng DNP) and for control, with 50 μL of HSA at 20 pg/μL (1 ng HSA), and after 10 min, placed on ice for β-hexosaminidase release assay.

OVA antigen: Same described method used for DNP antigen, but with overnight sensitization performed with murine post-immunization serum with OVA-specific IgE (0.25 μg/10^6 cells/mL) (anti-OVA IgE). For activation, 50 **m**L of OVA at 200 pg/μL (10 ng OVA) was used. For control, 50 μL of OVA at 200 pg/μL was added to cells without anti-OVA IgE overnight incubation.

3. Activation and rapid desensitization of RBL-2H3 cells to DNP

10^5 RBL-2H3 cells were seeded in 200 μL culture medium in flat-bottom 96-well plates and incubate for 3 hr to allow adhession. 0.005 μg of IgE-anti-DNP were added for a final concentration of 0.05 μg/mL. Cells were incubated overnight. Then cells were centrifuged for 5 min at 1000 rpm, in a centrifuge with a microtiter plate carrier. Medium was removed and 200 μL/well of *Tyrode's buffer* was added. Plates were centrifuged 5 min at 1000 rpm. Then, the supernatants were removed and plates were whased a second time to remove unbound IgE. Finally, 50 μL/well of Tyrode's buffer prewarmed to 37°C was added.

Desensitization protocol was started as per ***Table 3*** in one of the wells. At step 9, other wells were challenged with DNP-HSA or with HSA for control, or with Tyrode's buffer alone for determination of spontaneous release.

Ten minutes after the last desensitization step the plate was placed on ice. 70 μL of each supernatant was collected for further β-hexosaminidase determination. Then, 95 μL/well of cold Tyrode's buffer was added for pellet content determination.

To determine pellet content, 5 μL of 10% Triton X-100 were added to each welll and incubated 5 min at RT[a], occasionally pipetting up and down to lyse cells. Then the plate was centrifuged 10 min at 1000 rpm, 4°C. 70 μL of supernatant was collected for further β-hexosaminidase determination. β-hexosaminidase assay was performed as described in CHAPTER 1, section 4.

4. Specificity experiments

Cells were sensitized overnight with 0.25 μg/10^6 cells/mL of both anti-DNP IgE and anti-OVA IgE and then desensitized to DNP and challenged with DNP or OVA or desensitized to OVA and challenged with OVA or DNP.

5. Challenge with anti-IgE

After cells were desensitized or challenged with DNP or HSA, they were treated with 100 μg of rat anti-mouse IgE (clone R35-72 from BD Pharmingen). For control, cells incubated overnight with or without anti-DNP IgE were also treated with 100 μg of rat anti-mouse IgE.

6. Duration of desensitization

Cells were rapid desensitized as per ***Table 3***. After desensitization (nearly 2 h) cells were maintained for 10 min, 2 hours, or 4 hours at 37°C. After each time period, 1 ng of DNP-HSA or 25 mL of calcium ionophore A23187 (Sigma-Aldrich) 10 mM was added.

Non-desensitized cells were kept at 37°C and challenged with 1 ng of DNP-HSA or 1 ng HSA at the same time points as for desensitized cells. The total time for all cells at 37°C, since rapid desensitization protocol lasts nearly 2 h, was 6 h. Cell viability was assessed by trypan blue dye exclusion.

7. Statistical analysis

Data are expressed as mean ± SEM using Prism4. Statistical significance was determined using Student unpaired two-tailed *t* test. $P < 0.05$ was considered to be significant.

RESULTS

1. Protocol for DNP-HSA (1 ng) and OVA (10 ng) antigens

To induce hypo-responsiveness to IgE antigens, we sensitized mouse bone marrow-derived mast cells (mBMMC) with anti-DNP IgE and anti-OVA IgE and we delivered 11 incremental doses at 10-min intervals, for a total time of 100 min and for a total dose of 1 ng of DNP-HSA or 10 ng OVA. A protocol based on our previous publication (Morales AR et al.) was designed and used throughout the study (***Table 5***). The rapid desensitization protocol we present here is an *in vitro* protocol for mouse bone marrow-derived mast cells (mBMMC), consisting of delivering 11 incremental doses of antigen, DNP or OVA, at 10-min intervals in a cumulative fashion until the target dose (1 ng DNP or 10 ng OVA) is reached. This protocol is well described in Material and Methods Chapter 2, section 1.

Steps	Time (min)	Volume (μl)	Concentration (pg/μl)		Dose (pg)		
		DNP-HSA / OVA	DNP-HSA	OVA	DNP-HSA	OVA	
1	0	1	1	10	1	10	
2	10	1	5	50	5	50	
3	20	1	5	50	5	50	
4	30	1	10	100	10	100	
5	40	1	10	100	10	100	
6	50	2	10	100	20	200	
7	60	2	20	200	40	400	
8	70	4	20	200	80	800	
9	80	8	20	200	160	1600	
10	90	16	20	200	320	3200	
11	100	17.5	20	200	350	3500	
11 steps	100 min	54.5 μl*			1 ng	10 ng	Total

*added to 50 μL of cells

Table 5. *Rapid desensitization protocol in which eleven incremental doses of antigen DNP-HSA or OVA were delivered to mBMMCs at fixed time intervals until the target dose (1 ng DNP or 10 ng OVA) was reached.*

2. Establishment of controls

2.1. Step-by-step control with media without DNP-HSA

The first control named “Control Desens” consists of doing the desensitization protocol using media alone instead of antigen solutions and was done in parallel with the desensitization itself, adding the same volumes at each step. The only difference is the last step in which 17.5 μL of a 57.5 pg/μL solution is added to the tub, as explained in ***Table 6***.

Steps	Time (min)	Volume (μL) media	Concentration (pg/μL) DNP-HSA	Dose (pg) DNP-HSA
1	0	1	0	0
2	10	1	0	0
3	20	1	0	0
4	30	1	0	0
5	40	1	0	0
6	50	2	0	0
7	60	2	0	0
8	70	4	0	0
9	80	8	0	0
10	90	16	0	0
11	100	17.5	57.5	1000
11 steps	100 min	54.5 μL*		1 ng

*added to 50 μL of cells

Table 6. *"Control desens" in which the first ten steps are done in parallel with the regular desensitization protocol and using same volumes but with media alone. In the last step 17.5 μL of solution of 57.5 pg/μL is added to reach the target dose (1 ng DNP-HSA).*

This control was just used at the beginning of the study in order to check the desensitization protocol. Once the protocol was established and replicated, there was no need for it.

2.2 Control with HSA (DNP carrier) added to sensitized cells

This control named "HSA" consists in adding 50 μL of HSA solution (20 pg/μL) to an eppendorf containing 50 μL of IgE-anti-DNP-sensitized 10^6 cells, The final amount received by the cells is 1 ng HSA, compared to 1 ng DNP-HSA used for activation.

2.3 Control with DNP-HSA or OVA added to non-sensitized cells

This control named "No IgE+1ng DNP" consist in adding 50 μL of DNP-HSA solution (20 pg/μL) to an eppendorf containing 50 μL of non-sensitized 10^6 cells, for a total amount of 1 ng DNP-HSA.

This control was also used with the OVA system "No IgE+10ng OVA", adding 50 μL of OVA solution (200 pg/μL) to an eppendorf containing 50 μL of non-sensitized 10^6 cells, for a total amount of 10 ng OVA.

2.4 Comparison of controls

All controls used in this study are summarized in (***Figure 13***).

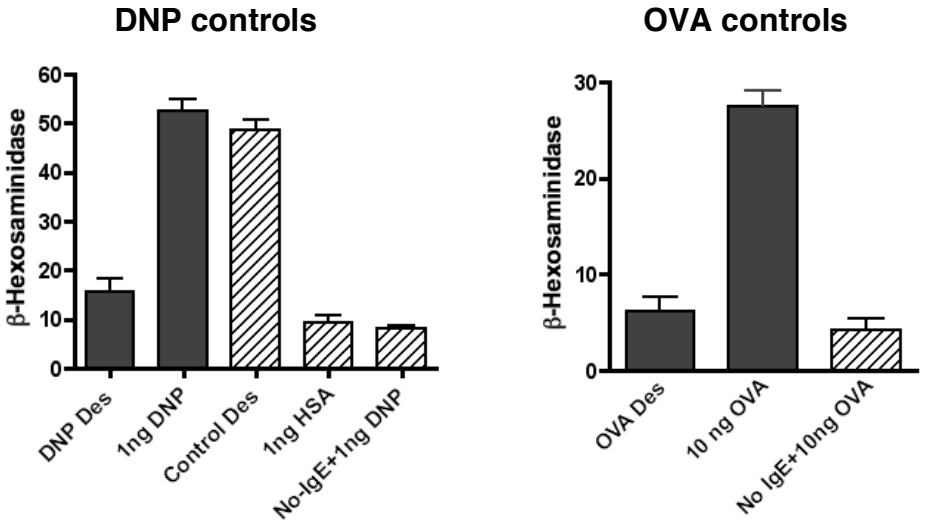


Figure 13. *β-hexosaminidase released from of mBMMCs with all controls used in this study. Rapid desensitization to 1ng DNP-HSA or 10ng OVA (DNP Des and OVA Des). Activation with 1ng DNP-HSA or 10 ng OVA (1ng DNP-HSA and 10ng OVA). Step-by-step control with media without DNP-HSA until the last step of the protocol where 1ng DNP-HSA was added (Control Des). Control with DNP carrier added to sensitized cells (1ng HSA). Control with DNP-HSA or OVA added to non-sensitized cells (No IgE+1ng DNP and No IgE+10ng OVA).*

3. Sensitization assays

Early studies of IgE antibody binding to the FcεRI receptor (MacGlashan DJr et al. (1)), showed that unoccupied receptors on human basophils should be fully sensitized following a 20 min incubation with 10 pg/ml of anti-DNP IgE at 37°C. But posterior studies showed that DNP-HSA-induced histamine release following this previous sensitization protocol was rather poor and that sensitizing mast cells for 2 days with 10 pg/ml IgE antibody induced much better histamine release (Hsu C et al.). Thus, BMMC response to antigenic challenge does improve if sensitization is prolonged for several hours.

In order to test our cells (mBMMCs cultured in IL-3 enriched media) and our system (activation and desensitization of 10^6 BMMCs), and taking on account a previous study done by our group (Morales AR et al.), two different experiments were done.

A first one, to establish the amount of anti-DNP IgE used for sensitization, and a second one, to show how anti-DNP IgE incubation time affects our system.

3.1 Amount of anti-DNP IgE

In the first publication of our group (Morales AR et al.), 0.25 μg of anti-DNP IgE were used for cells sensitization. We were concerned about the optimal amount of IgE to achieve an optimal receptor occupancy, therefore we doubled this amount to check if better results could be obtained. 10^6 BMMCs were sensitized overnight with 0.25 μg or 0.50 μg of anti-DNP IgE and DNP-HSA desensitized (DNP Des), DNP-HSA activated (1 ng DNP) and HSA challenged (1 ng HSA) as a control (***Figure 14***).

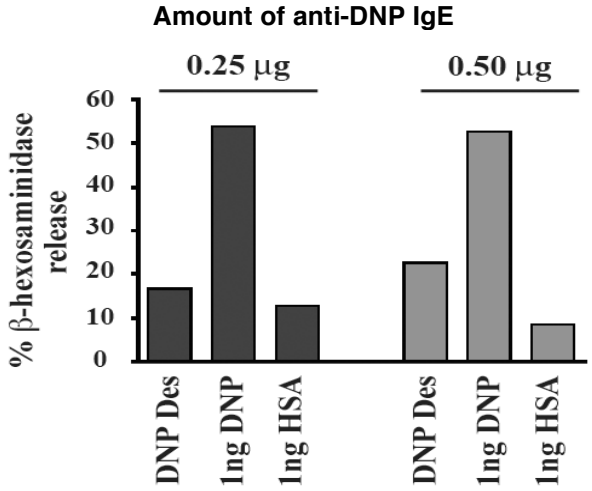


Figure 14. *β-hexosaminidase released from* 10^6 *mBMMCs overnight incubated with 0.25 μg or 0.50 μg of anti-DNP IgE per ml and then rapidly desensitized (DNP Des), challenged with 1 ng DNP-HSA (1 ng DNP) or challenged with 1 ng HSA (1 ng HSA).*

Based on the results, our best window between desensitization and activation was accomplished using 0.25 μg of anti-DNP IgE as sensitization amount and this is the one used over the course of this study, unless specifically pointed.

3.2 Time of incubation with anti-DNP IgE

In order to know the best sensitization time for our system, 10^6 BMMCs were sensitized for three different periods of time: 24, 16 and 3 hours with 0.25 μg of anti-DNP IgE. Cells were DNP-HSA desensitized (DNP Des), DNP-HSA activated (1 ng DNP) and HSA challenged (1 ng HSA) as a control.

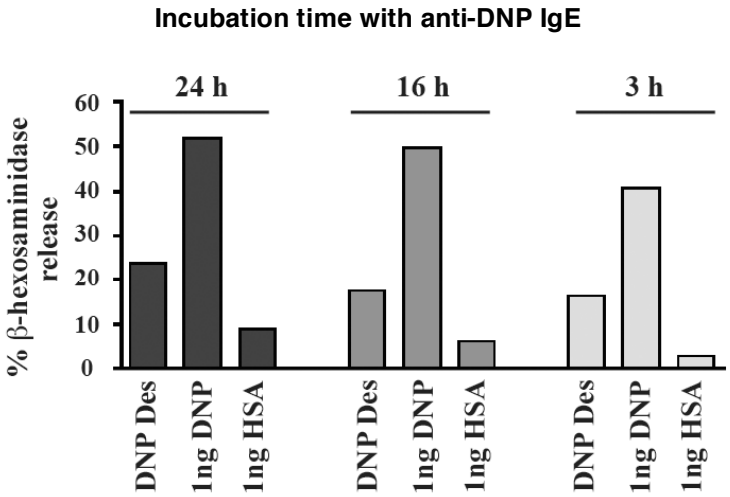


Figure 15. *β-hexosaminidase released from mBMMCs incubated with 0.25 μg of anti-DNP IgE for 3h, 16h or 24h and then rapidly desensitized (DNP Des), challenged with 1 ng DNP-HSA (1 ng DNP) or challenged with 1 ng HSA (1 ng HSA).*

Based on the results (***Figure 15***), our best window between desensitization and activation was accomplished with 16 hours of incubation with 0.25 μg of anti-DNP IgE. Thus, overnight sensitization is used all over the course of this research study.

4. Dose-response curve to DNP-HSA

In order to compare single dose antigen delivery (activation) with sequential cumulative doses (rapid desensitization), we first assessed the dose response curve to DNP-HSA antigen, by β-hexosaminidase release, with cells sensitized with anti-DNP IgE (***Figure 16***).

DNP-HSA doses of 1, 5 and 10 pg were non-activating as single doses, since the percentage of β-hexosaminidase release was comparable to that of the control (1 ng HSA, lower white bar in ***Figure 16***). Cells challenged with higher doses of antigen (>10 pg DNP-HSA) delivered as single doses achieved significant β-hexosaminidase release.

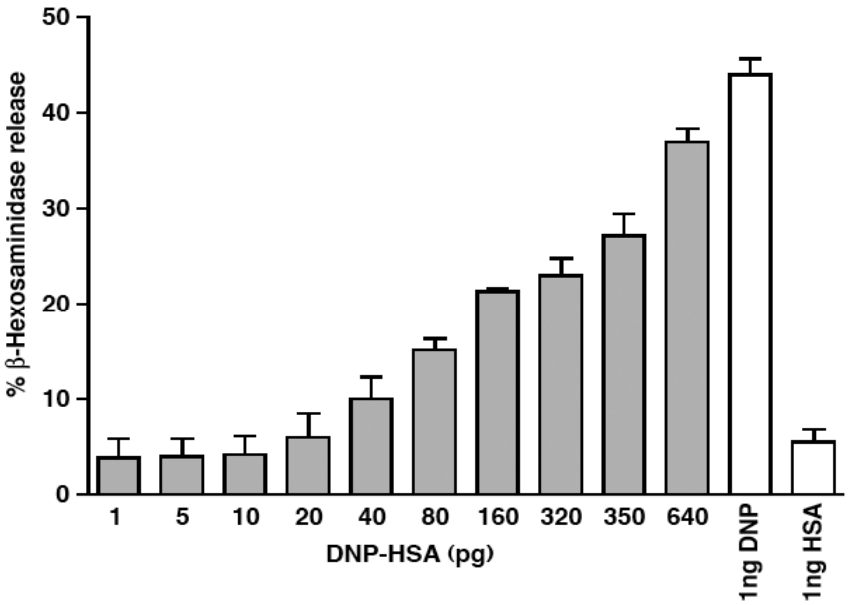


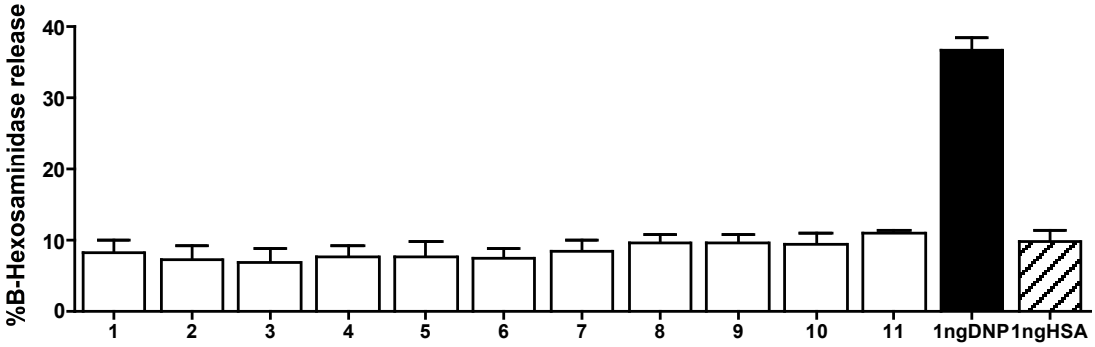
Figure 16. *Dose response curve (single dose additions). β-hexosaminidase released from 10^6 mBMMCs, overnight incubated with 0.25 μg of anti-DNP, challenged with increasing single doses of DNP-HSA. 1 ng DNP and 1 ng HSA are used as controls.*

Values of β-hexosaminidase release were dose-dependent, achieving the highest value at 1 ng DNP-HSA (1 ng DNP, higher white bar in ***Figure 16***), which represents the optimal triggering dose used as target dose for rapid desensitization.

5. Antigen doses added sequentially induce hypo-responsiveness

The release obtained with single-dose additions (***Figure 16***) was compared to that obtained with doses added sequentially, following every step of the desensitization protocol (***Figure 17*** white bars). White bars represent β -hexosaminidase released at each particular point in the cumulative sequence of antigen additions.

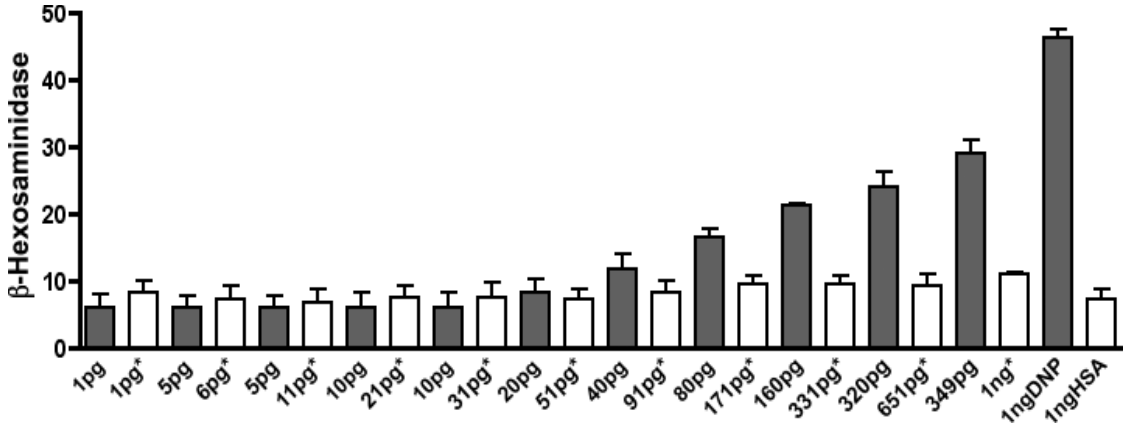
A maximum of 10% β-hexosaminidase release was achieved at all points in the sequence, showing that the desensitization process did not induce a slow release of mediators.



Time (min)	1	2	3	4	5	6	7	8	9	10	11
0	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg
10	...	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg
20	...	...	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg
30	...	...	...	10pg	10pg	10pg	10pg	10pg	10pg	10pg	10pg
40	...	...	...	...	10pg	10pg	10pg	10pg	10pg	10pg	10pg
50	...	...	...	...	...	20pg	20pg	20pg	20pg	20pg	20pg
60	...	...	...	...	...	...	40pg	40pg	40pg	40pg	40pg
70	...	...	...	...	...	...	...	80pg	80pg	80pg	80pg
80	...	...	...	...	...	...	...	...	160pg	160pg	160pg
90	...	...	...	...	...	...	...	...	...	320pg	320pg
100	...	...	...	...	...	...	...	...	...	...	349pg
Total dose	**1pg**	**6pg**	**11pg**	**21pg**	**31pg**	**51pg**	**91pg**	**171pg**	**331pg**	**651pg**	**1ng**

Figure 17. *Sequentially added doses. Accumulation of β-hexosaminidase at each particular point in the sequence of DNP-HSA additions (DNP in the graph) as per protocol in* ***Table 5****. Experiments were done with 10^6 mBMMCs overnight incubated with 0.25 μg of anti-DNP IgE. Data are expressed as mean ± SEM of three independent experiments.*

The release obtained with single-dose additions in ***Figure 16*** and that obtained with doses added sequentially in ***Figure 17***, is compared in ***Figure 18***. Sequentially added doses in a cumulative fashion induce mast cell hyporesponsiveness as shown by lack of degranulation.



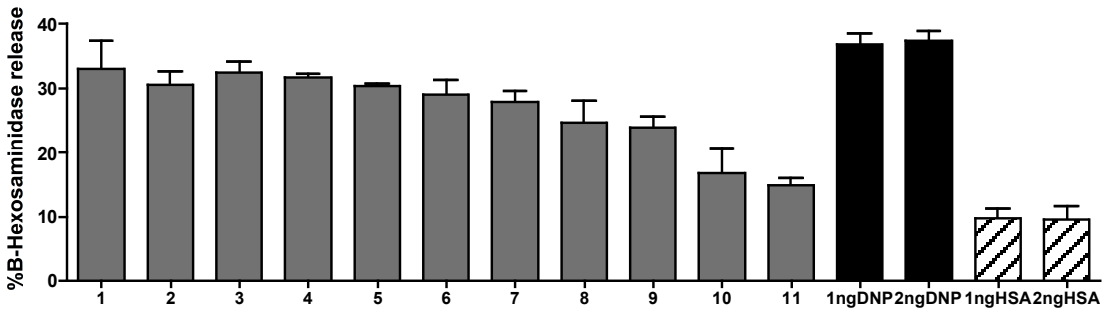
Total dose (single doses)	1pg	5pg	5pg	10pg	10pg	20pg	40pg	80pg	160pg	320pg	349pg
Total dose* (sequential doses)	1pg*	6pg*	11pg*	21pg*	31pg*	51pg*	91pg*	171pg*	331pg*	651pg*	1ng*

***Figure 18**. β-hexosaminidase release obtained with single dose challenges (grey bars) versus sequentially added doses (white bars). Experiments were done with 10^6 mBMMCs overnight incubated with 0.25 μg of anti-DNP IgE. Data are expressed as mean ± SEM of three independent experiments.*

6. Achievement of hypo-responsiveness

It is not known whether human desensitizations are achieved through a threshold dose or if the process required all doses. To determine whether there was a threshold dose that initiated hypo-responsiveness, cells that were treated with sequentially added doses in a cumulative fashion, were followed by a triggering dose of 1 ng of DNP-HSA (***Figure 19***).

At each cumulative dose followed by a triggering dose of 1 ng DNP-HSA, inhibition of activation was dose-dependent, with the greatest inhibition at the highest cumulative dose.



Time (min)	1	2	3	4	5	6	7	8	9	10	11
0	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg	1pg
10	**1ng**	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg
20	…	**1ng**	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg	5pg
30	…	…	**1ng**	10pg	10pg	10pg	10pg	10pg	10pg	10pg	10pg
40	…	…	…	**1ng**	10pg	10pg	10pg	10pg	10pg	10pg	10pg
50	…	…	…	…	**1ng**	20pg	20pg	20pg	20pg	20pg	20pg
60	…	…	…	…	…	**1ng**	40pg	40pg	40pg	40pg	40pg
70	…	…	…	…	…	…	**1ng**	80pg	80pg	80pg	80pg
80	…	…	…	…	…	…	…	**1ng**	160pg	160pg	160pg
90	…	…	…	…	…	…	…	…	**1ng**	320pg	320pg
100	…	…	…	…	…	…	…	…	…	**1ng**	349pg
110	…	…	…	…	…	…	…	…	…	…	**1ng**
Total dose	**1.001pg**	**1.006pg**	**1.011pg**	**1.021pg**	**1.031pg**	**1.051pg**	**1.091pg**	**1.171pg**	**1.331pg**	**1.651pg**	**2ng**

Figure 19. *Responsiveness of different DNP-HSA challenged BMMC to 1 ng DNP-HSA that was added 10 min after the last DNP-HSA addition in the sequence of DNP-HSA additions as per protocol in* ***Table 5****. Experiments were done with 10^6 mBMMCs overnight incubated with 0.25 μg of anti-DNP IgE. Data are expressed as mean ± SEM of three independent experiments.*

In ***Figure 20*** there is a comparation between the β -hexosaminidase release from cells with sequentially added doses (white bars) and the release obtained in a replicated tub with a 1ng DNP-HSA challenge 10 min after the last DNP-HSA addition in the sequence of DNP-HSA additions as per the rapid desensitization protocol (grey bars).

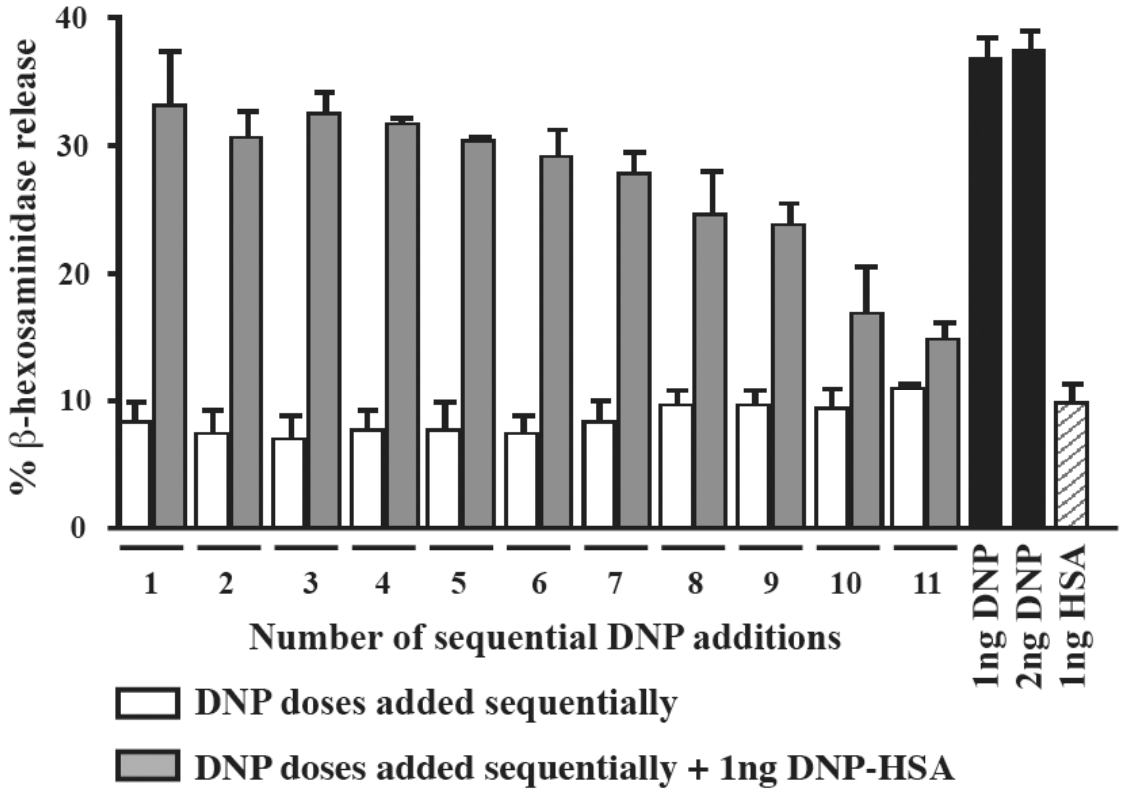


Figure 20. *White bars show accumulation of β-hexosaminidase at each particular point in the sequence of DNP-HSA additions (DNP in the graph) as per protocol in* ***Table 5****. Grey bars show replicate samples in which 1 ng DNP-HSA was added 10 min after the last DNP-HSA addition in the sequence of DNP-HSA additions as per protocol in* ***Table 5****.*

Response to the triggering dose declined with increasing number of sequential doses and the greatest hypo-responsiveness was achieved with the highest number of sequential additions (11, in ***Figure 20***) indicating that at each additional dose hypo-responsiveness increases but is not accomplished until the end of the desensitization protocol. Thus, all doses of the protocol are needed in order to achieve hypo-responsiveness.

7. Hypo-responsiveness with increase activating doses is maintained after rapid desensitization

To test whether cells hypo-responsiveness achieved with rapid desensitization could be overcome with higher challenging doses, we analyzed the response of desensitized cells to 1ng DNP-HSA (DNPDes in ***Figure 21***) to activating doses of 1, 2, 3, 4 and 5 ng of DNP-HSA. Up to 5-fold increase in challenging dose did not reverse desensitization.

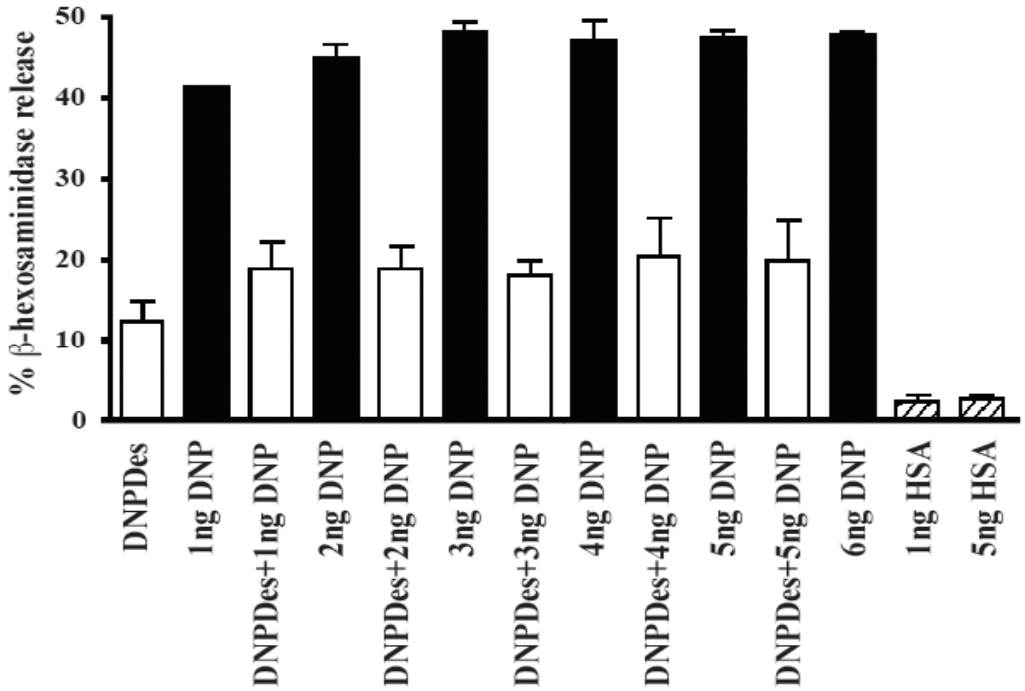


Figure 21 *Responsiveness of desensitized BMMC to 1ng DNP-HSA (DNPDes) to different DNP-HSA challenge doses (1, 2, 3, 4 and 5 ng of DNP-HSA). Experiments were done with 10^6 mBMMCs overnight incubated with 0.25 μg of anti-DNP IgE. Data are expressed as mean ± SEM of three independent experiments.*

8. Validation of the rapid desensitization protocol

Our understanding of IgE desensitizations has been hampered by the lack of valid *in vitro* mast cell models to provide quantitative and qualitative insight into the process. We wanted to validate our protocol from two different perspectives: first, showing that our protocol for rapid desensitization can be used with different target doses and second, using the protocol with a different cell type, a rat basophilic leukemia (RBL-2H3) cell line.

8.1 Desensitization with different target dose: 1, 5, 10 ng DNP-HSA

Our protocol was effective when increasing the target dose to 5 and 10 ng (***Figure 22***), with the same number of steps, time between steps and starting dose (1/1000 the target dose).

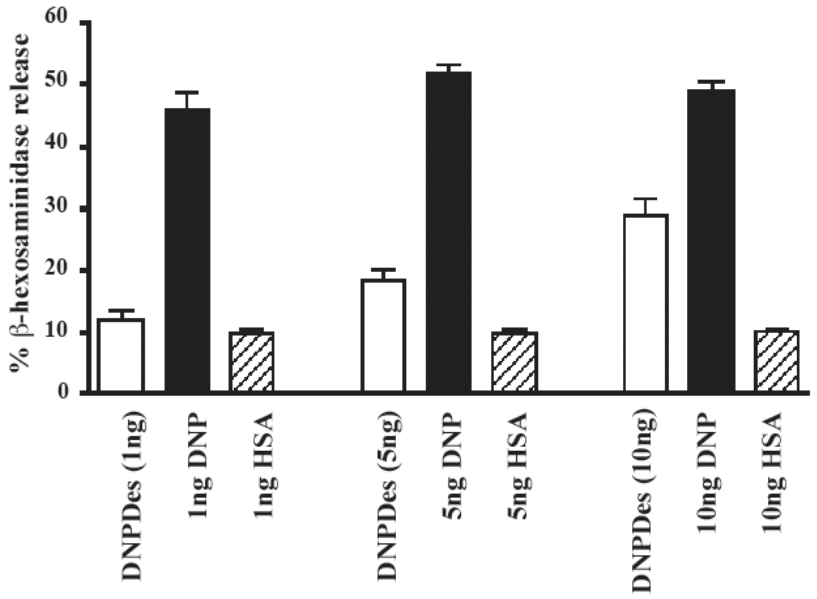


Figure 22. *Rapid desensitizations to 1, 5 and 10 ng of DNP-HSA with their correspondent controls for DNP-HSA and HSA challenges of 1, 5 and 10 ng. Experiments were done with 10^6 mBMMCs overnight incubated with 0.25 μg of anti-DNP IgE. Data are expressed as mean ± SEM of three independent experiments.*

Cells desensitized to 1 ng DNP-HSA showed a 75% inhibition whereas cells desensitized to 5 and 10 ng DNP-HSA had a 65% and 41% inhibition of β-hexosaminidase release, respectively. Thus the higher the target dose, the lower the level of desensitization shown by a higher amount of β-hexosaminidase release.

8.2 Desensitization with different cell type: RBL-2H3

RBL 2H3 cell line has been used to examine signal transduction events in FcεRI mediated mast cell activation. Thus, they are a good cell line to validate our protocol.

RBL 2H3 cells were senswitized overnight with 0.005 μg of anti-DNP IgE and rapidly desensitized to 1ng DNP-HSA following the protocol in ***Table 5***. Cells were also challenged with 1ng DNP-HSA or 1ng HSA as detailed in CHAPTER 2 Section 3. Rapidly desensitized cells showed a 55% inhibition of β-hexosaminidase release compared to cells activated with the same amount of DNP-HSA (***Figure 23***).

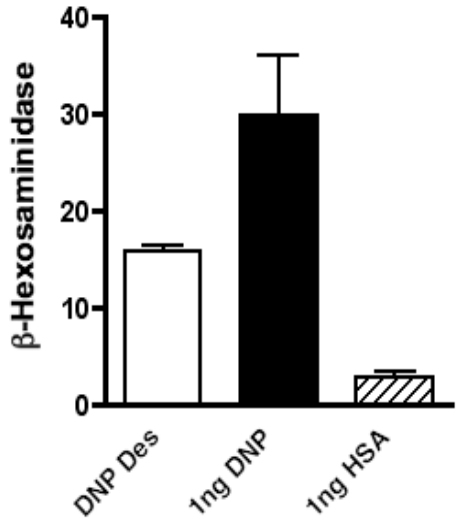


Figure 23. *β-hexosaminidase released from RBL-2H3 cells overnight incubated with 0.005 µg of anti-DNP IgE and rapidly desensitized (DNP Des), challenged with 1 ng DNP-HSA (1 ng DNP) or challenged with 1 ng HSA (1 ng HSA). Experiments were done with 10^5 cells per sample. Data are expressed as mean ± SEM of three independent experiments.*

This result shows that even with an adhesive cell line and adapting the methodology in accordance, our rapid desensitization protocol is valid for two different cell types.

9. Rapid desensitization impairs early-phase activation responses in BMMCs

9.1 Degranulation

Mast cells release multiple classes of enzymes such as β-hexosaminidase and TNFα, a preformed cytokine, as a part of the early-phase response that occurs within minutes after the antigen cross-linking of the IgE/FcεRI complex, in the process of degranulation.

9.1.1 β-hexosaminidase release assay

BMMC sensitized with anti-DNP IgE or anti-OVA IgE, were rapidly desensitized as per the protocol presented in ***Table 5***. In both DNP and OVA systems, we measured the release of β-hexosaminidase when antigen was delivered as a single dose (1 ng DNP-HSA / 10 ng OVA, black bars in ***Figure 24***) or when antigen was delivered following the rapid desensitization protocol (white bars in ***Figure 24***). Negative controls were 1ng HSA or No IgE+10 ng OVA (dashed bars in ***Figure 24***).

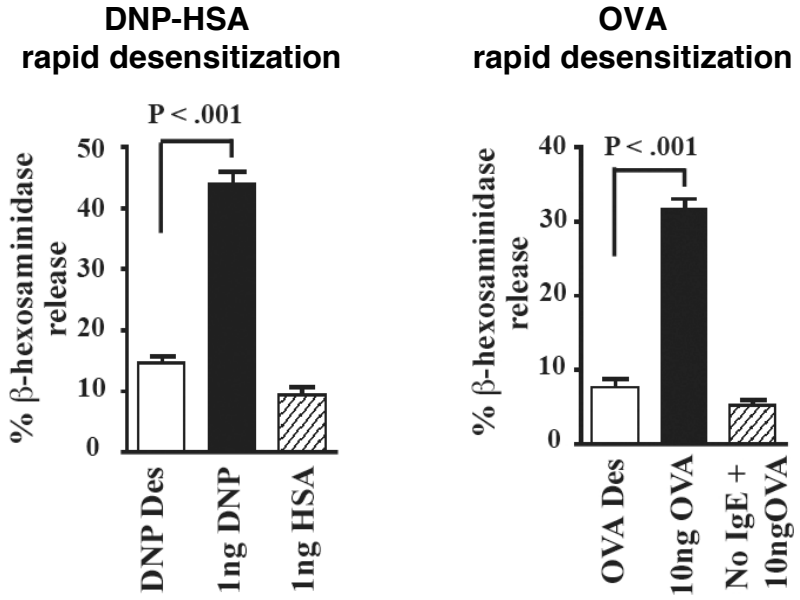


Figure 24. *β-hexosaminidase release from 10^6 mBMMCs overnight incubated with 0.25 μg of anti-DNP IgE, after DNP or OVA desensitization (DNPDes or OVADes) or DNP-HSA or OVA challenge (1 ng DNP or 10 ng OVA). Negative controls were 1ng HSA or No IgE+10 ng OVA. Data are expressed as mean ± SEM of three independent experiments.*

Cells desensitized to 1 ng DNP-HSA showed a 78% inhibition of β-hexosaminidase compared to cells activated with the same amount of DNP-HSA (1 ng) and cells desensitized to 10 ng OVA showed a 71% reduction in β-hexosaminidase release compared to cells activated with the same amount of OVA (10 ng).

These results demonstrate that rapid desensitization inhibits degranulation as seen by β-hexosaminidase release assay.

9.1.2 Analysis of pre-formed TNF-α

TNF-α is a preformed proinfammatory cytokine released from mast cells upon degranulation. Pre-formed TNF-α is released upon IgE stimulation in the early-phase response, while secretion of de novo synthesized TNF-α and IL-6 production occurs several hours post-stimulation, in the late-phase response.

Pre-formed TNF-α released after 1 ng DNP-HSA challenge was 490 ± 52 pg, while in rapidly desensitized cells the release was 185 ± 35 pg, as shown in ***Figure 25.***

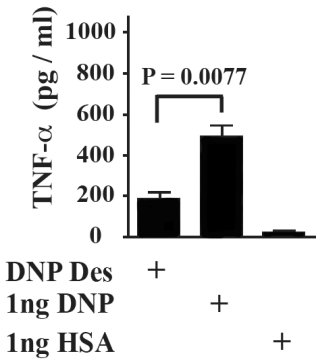


Figure 25. *Amount of pre-formed TNF-α released from 10^6 mBMMCs overnight incubated with 0.25 μg of anti-DNP IgE, 30 min after being rapidly desensitized, challenged with 1 ng DNP-HSA or challenged with 1 ng HSA as control. Data are expressed as mean ± SEM of three independent experiments.*

There is a significant 62% reduction in pre-formed TNF-α released by rapidly desensitized cells compared to 1 ng DNP-HSA challenge. This result demonstrates that desensitization impairs degranulation, as seen by Elisa quantification of the preformed TNF-α store in granules.

9.2 Calcium mobilization assay

Exocytosis of pre-formed mediators from granules cannot occur without external calcium entry. During mast cells activation, there is a Ca^{2+} influx across the plasma membrane, but this calcium entry requires the depletion of intracellular calcium stores in the endoplasmic reticulum (ER) (***Figure 26***).

Depletion of the ER stores leads to activation of plasma membrane calcium-release activated calcium (CRAC) channels that allow Ca^{2+} entry, through the interaction of the ER Ca^{2+} sensor, stromal interacting molecule-1 (STIM1), with a unique Ca^{2+} channel protein, Orai1/CRACM1, acting as a modulator with a channel pore highly selective for Ca^{2+} (Vig M et al. / Ma HT et al.).

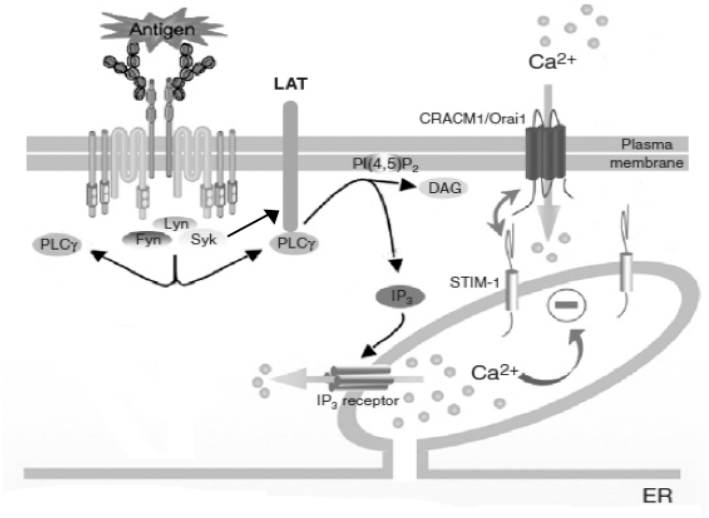


Figure 26. *Schematic representation of calcium mobilization after antigen crosslinking of FcεRI receptors.* ***Adapted from Rivera J et al.***

We compared changes in fluorescence ratios when a triggering dose of 1 ng DNP-HSA was added to five different cell samples. Calcium flux was observed in non-desensitized cells after antigen addition, **(1)** in ***Figure 27***, but a 90 % inhibition of extracellular calcium influx was observed when the antigen was added to desensitized cells **(2)** and, as expected, to cells that were not sensitized with anti-DNP IgE **(3)**. The last two samples represent how an addition of EGTA, a calcium chelator, before DNP-HSA challenge of non-desensitized cells and of rapidly desensitized cells, can affect calcium mobilization. Chelation of extracellular calcium with EGTA provided a lower intracellular calcium response in desensitized cells (light grey in ***Figure 27***) as compared to activated cells (dark grey in ***Figure 27***). The inhibition of calcium influx was paralleled by the inhibition of degranulation.

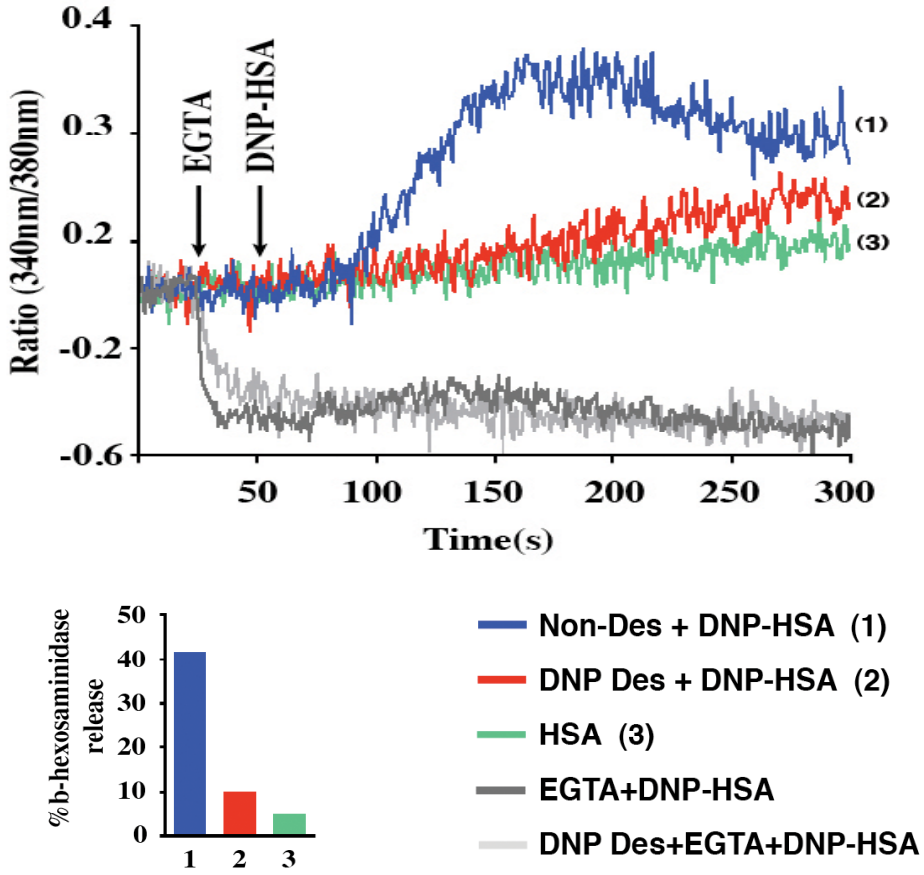


Figure 27. *Rapid desensitization to DNP-HSA prevents calcium influx. Graph shows calcium mobilization with and without EGTA addition (at 25 seconds) before antigen addition (at 50 seconds). Columns show β - hexosaminidase release in a parallel experiment. The numbers on the right of the graph correspond to numbers in the bar graph and in the legend. Data are representative of four independent experiments.*

DNP-desensitized cells showed 90% inhibition of calcium mobilization, indicating that calcium-dependent events are possibly impaired during desensitization, and because desensitized cells had a lower EGTA calcium response than activated cells, it is possible that a threshold calcium signal cannot be reached during desensitization, which may disable CRAC channels preventing degranulation.

9.3 Analysis of arachidonic acid metabolites: LTC4, LTB4 and 12-HHT

In addition to degranulation, rapidly synthesized lipid mediators such as leukotrienes (LTs) and prostaglandins are released within a short time after activation. Because calcium mobilization is key to arachidonic acid metabolization and generation of prostaglandins and leukotrienes, we studied some of the products of the arachidonic acid metabolization (***Figure 28***).

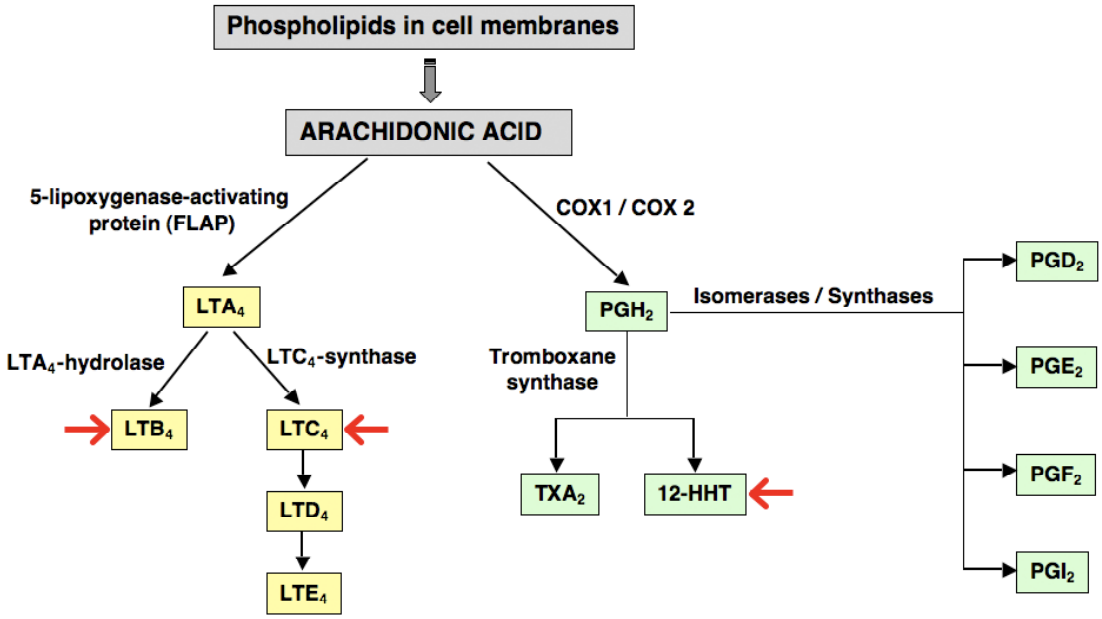


Figure 28. *Simplified flowchart of the arachidonic acid metabolism. Red arrows point out the products of the arachidonic acid metabolization measured in this study.*

Thirty minutes after 1 ng DNP-HSA challenge, cell supernatants was analysed by reverse-phase high-performance liquid chromatography (RP-HPLC); cysteinyl leukotriene C4 (LTC_4), leucotriene B4 (LTB_4), and 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid (12-HHT) were identified with retention times of 21.4, 23.7, and 24.4 min, respectively, with prostaglandin B2 (PGB_2) as an internal standard.

LTB_4, LTC_4, and 12-HHT were not detected in rapidly desensitized cell supernatants or in cells treated with 1 ng HSA as seen in ***Figure 29***. This result indicates a lack of arachidonic acid metabolization with desensitization.

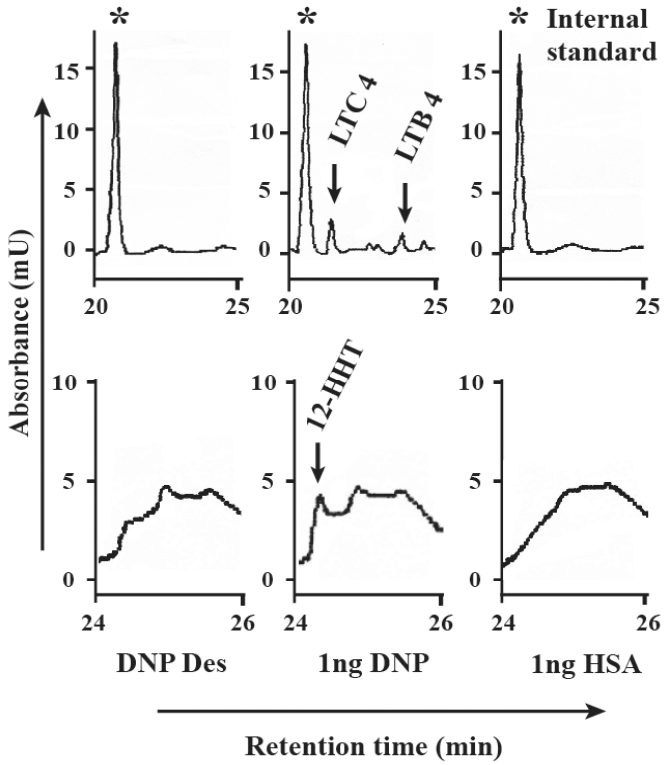


Figure 29. *RP-HPLC analysis of arachidonic acid products in supernatants of cells treated as indicated, with retention times for PGB2 (internal standard), LTC4, LTB4, and 12-HHT of 20.6, 21.4, 23.7, and 24.4 min, respectively. Data are representative of four independent experiments.*

9.4 Phosphorylation of several signal molecules

9.4.1 Analysis of signal transducer and activator of transcription 6 (STAT6)

It has been reported that STAT6 plays a pivotal role in antigen/IgE/FcεRI-mediated cytokine release from mast cells and that STAT6 phosphorylation occurs not only through the JAK-STAT pathway after IL-4 receptor activation but also after antigen cross-linking of FcεRI/IgE (Malaviya R et al.).

Since our previous studies showed that STAT6-null BMMCs from BALB/c and C57BL/6 mice could not be desensitized (Morales AR et al.), we explored how rapid desensitization targeted STAT6. We evaluated STAT6 phosphorylation in

DNP-HSA-activated and desensitized cells and observed that STAT6 was not phosphorylated with rapid desensitization, as seen in ***Figure 30***.

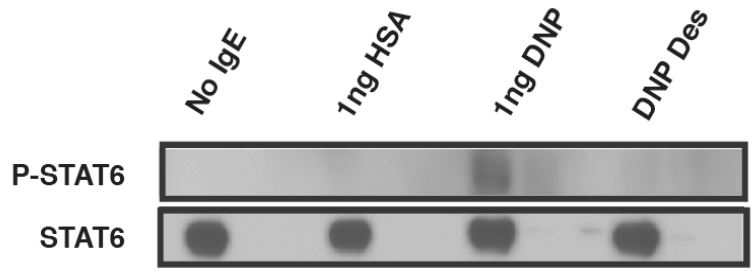


Figure 30. *Rapid desensitization prevents STAT6 phosphorylation. Cell lysates from anti-DNP IgE-sensitized BMMCs treated as indicated were analyzed with anti-phospho-STAT6 Ab and reblotted with anti-STAT6 Ab. Controls: 1 ng HSA challenge and non IgE treated cells (No IgE). Data are representative of three independent experiments.*

9.4.2 Analysis of linker for the activation of T cells (LAT)

Following FcεRI/IgE aggregation and cross-linking by antigen, Lyn phosphorylation of β and γ chains is followed by Syk and Fyn phosphorylation. LAT, which is phosphorylated by Syk, serves as a docking site for further events leading to degranulation (Kraft S et al.) and LAT-deficient BMMC have impaired calcium mobilization and granule mediator release (Saitoh S et al. (1)).

We detected LAT phosphorylation during activation but not during rapid desensitization (***Figure 31***).

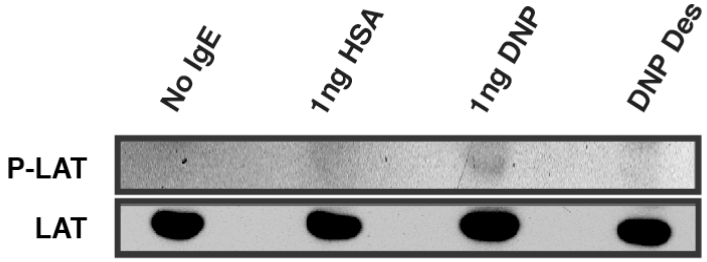


Figure 31. *Rapid desensitization prevents LAT phosphorylation. Cell lysates from anti-DNP IgE-sensitized BMMCs treated as indicated were analyzed with anti-phospho-LAT Ab and reblotted with anti-LAT Ab. Controls: 1 ng HSA challenge and non IgE treated cells (No IgE). Data are representative of three independent experiments.*

9.4.3 Analysis of p38 mitogen-activated protein kinase (p38 MAPK)

Since FcεRI-mediated mitogen-activated protein kinases (MAPKs) activation leads to gene transcription of several cytokines (Kalesnikoff J et al. / Ishizuka T et al.), we next examined the levels of phosphorylation of p38 MAPK in DNP-HSA-activated and desensitized cells (***Figure 32***).

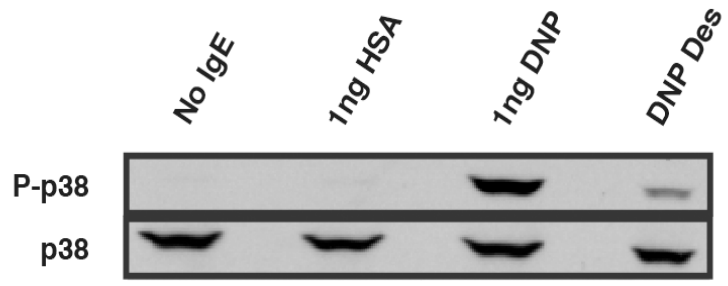


Figure 32. *Rapid desensitization prevents p38-MAPK phosphorylation. Cell lysates from anti-DNP IgE-sensitized BMMCs treated as indicated were analyzed with anti-phospho-p38 Ab and reblotted with anti-p38 Ab. Controls: 1 ng HSA challenge and non IgE treated cells (No IgE). Data are representative of three independent experiments.*

As expected by the low levels of TNF-α and IL-6 production (see following section 10), p38 MAPK phosphorylation was inhibited by rapid desensitization.

Overall, this lack of phosphorylation of signal molecules in the activation cascade suggest that mast cell signal transduction is blocked at an early step preventing phosphorylation of STAT6 and LAT and that molecular events such as p38 MAP kinase activation leading to cytokine gene transcription, were also inhibited during rapid desensitization.

10. Rapid desensitization impairs late activation responses in BMMC: IL-6 and newly generated TNF-α

Within hours after activation and as a part of the late-phase response, mast cells produce large amounts of various cytokines and chemokines. As seen in section 9.1.2, pre-formed TNF-α is released upon IgE stimulation in the early-phase response, but there is secretion of de novo synthesized TNF-α in the late-phase response, as well as IL-6 production that occur several hours post-stimulation.

Because early-phase activation events may influence late-phase responses, and because desensitization may affect early and late-phase responses differently, we studied TNF-α, a product of mast cell responses in both phases, and IL-6, a cytokine not typically stored but produced in the late phase.

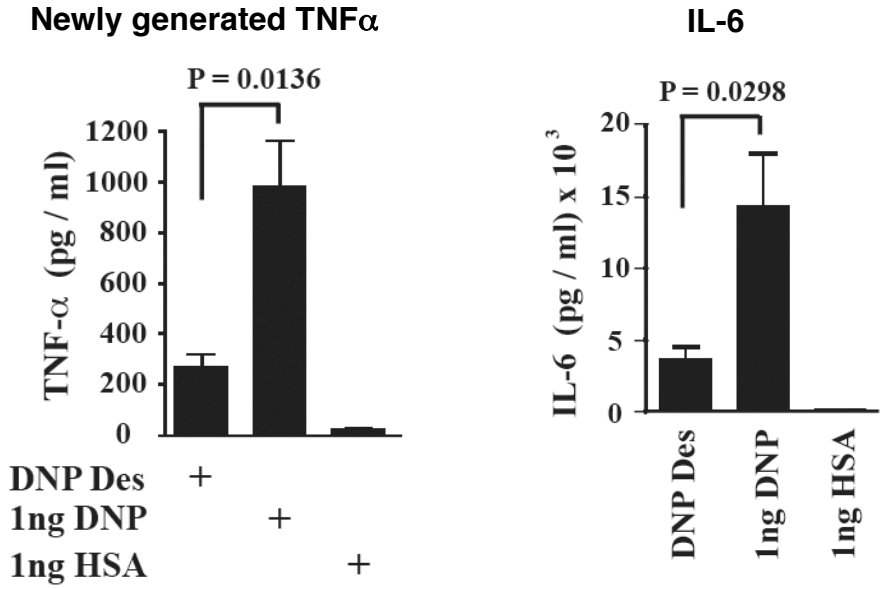


Figure 33. *Release of IL-6 and newly generated TNFα. Levels were estimated in cell-free supernatants of mBMMC by ELISA. Cell supernatants were collected 4 h after rapid desensitization, after challenge with 1 ng DNP-HSA and after challenge with 1 ng HSA as control.*

During the late phase response, 4 hours after activation or desensitization, the release of newly generated TNF-α from DNP-activated cells was 978 ± 178 pg/ml, while rapidly desensitized cells released 272 ± 47 pg/ml, which consists in a significant 72% reduction (***Figure 33***). The production of IL-6 assessed 4 hours after activation or desensitization was 14,362 ± 3,590 pg/ml and 3,665 ± 843 pg/ml, respectively, showing a 75% reduction (***Figure 33***).

These results, together with results from section 9.1.2, indicate that desensitization impairs the release of pre-formed (30 minutes) and newly generated TNFα (4 hours) and this can be compared in ***Figure 34.***

Pre-formed and newly generated TNFα

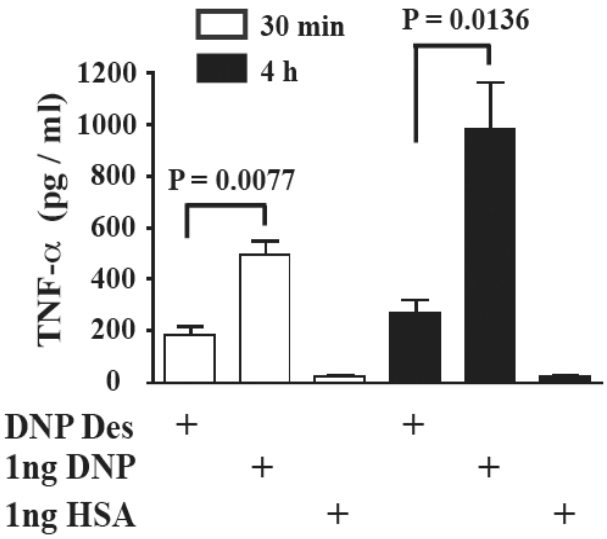


Figure 34. *Release of pre-formed (30 min) and newly generated (4 h) TNFα. Cell-free supernatants from 10^6 mBMMCs were collected 30 min and 4 h after rapid desensitization, after challenge with 1 ng DNP-HSA and after challenge with 1 ng HSA as control. TNFα levels were estimated by ELISA. Data are expressed as mean ± SEM of three independent experiments*

11. Duration of hypo-responsiveness after desensitization

Because the duration of desensitization may depend on the presence of bound and soluble antigen we determined the duration and antigen requirements for maintaining hypo-responsiveness after desensitization.

Cells challenged with 1 ng DNP-HSA at 10 min, 2 h, and 4 h after desensitization, remained hypo-responsive with a 20% β-hexosaminidase release (***Figure 35***, first bar of each time group of bars). Treatment of desensitized cells with ionomycin (A23187, Sigma-Aldrich) at 10 min, 2 h, or 4 h after desensitization, resulted in high levels of β-hexosaminidase release (***Figure 35***, second bar of each time group of bars), indicating that desensitized cells were not mediator-depleted.

Further time points were not pursued due to diminishing cell viability (from 91% to 83%) after nearly 6 h (100 min during desensitization and then 4 h). This decrease in cell viability was attributed to low volume (10^6 cells in 50 to 100 µL) and IL-3 and CO_2 depletion.

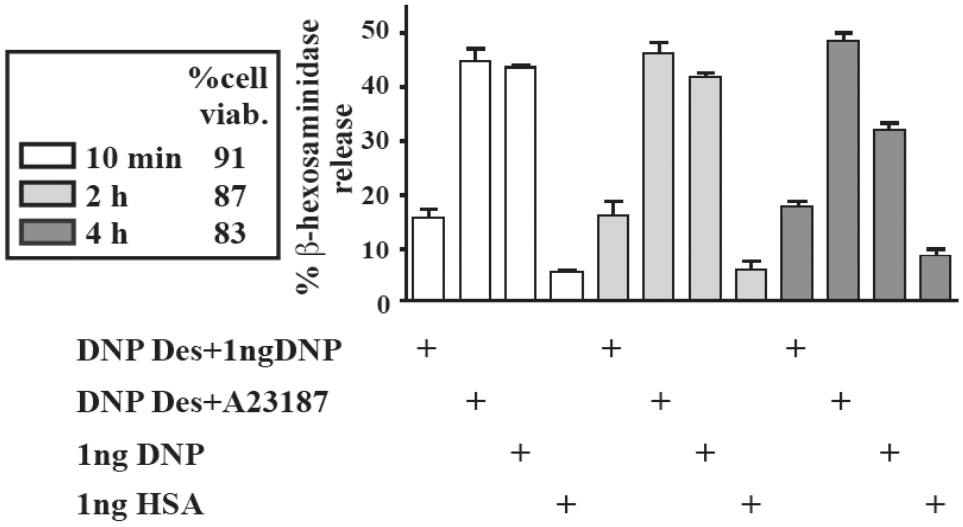


Figure 35. *Duration of hypo-responsiveness after rapid desensitization. % β -hexosaminidase release from cells sensitized overnight with anti-DNP IgE in response to the indicated treatments at various time points (10 min, 2 h and 4 h) after desensitization. Data are expressed as mean ± SEM of three independent experiments.*

We then considered the possibility that desensitized BMMCs could remain hypo-responsive to further stimulation due to the excess of soluble antigen. Washed and non-washed desensitized cells responded similarly to antigen challenge (***Figure 36***), indicating that once hypo-responsiveness was achieved, the presence of soluble antigen was not required for maintaining desensitization.

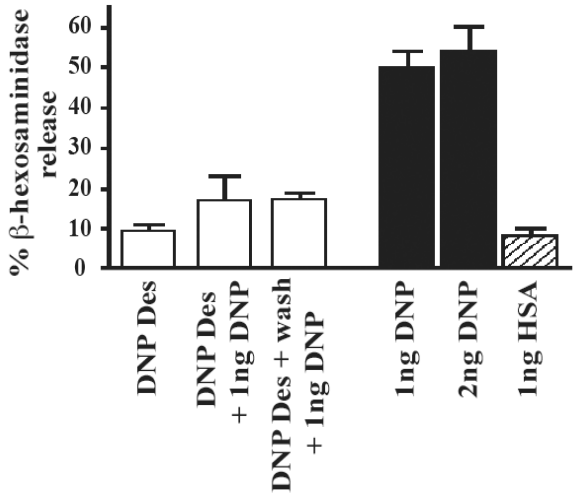


Figure 36. *Antigen requirements for hypo-responsiveness after rapid desensitization. Maintenance of desensitization with or without washing before challenge with 1 ng DNP-HSA. Data are expressed as mean ± SEM of three independent experiments*

12. Inhibition of FcεRI internalization

12.1 Surface expression of FcεRI and IgE anti-DNP

Internalization of antigen/IgE/FcεRI complexes has been demonstrated after cell activation (Fattakhova GV et al. / Mao SY et al.), and it has been suggested that mast cell hyporesponsiveness to low antigen doses is due to internalization of antigen-bound receptors (Shalit M et al.).

We wanted to determine the fate of the antigen/IgE/FcεRI complex with desensitization. Thus we analyzed surface expression of FcεRIα and IgE anti-DNP in rapidly desensitized cells, in cells challenged with 1 ng DNP-HSA or with 1ng HSA, and in non-sensitized cells (***Figure 37*** and ***Figure 38***).

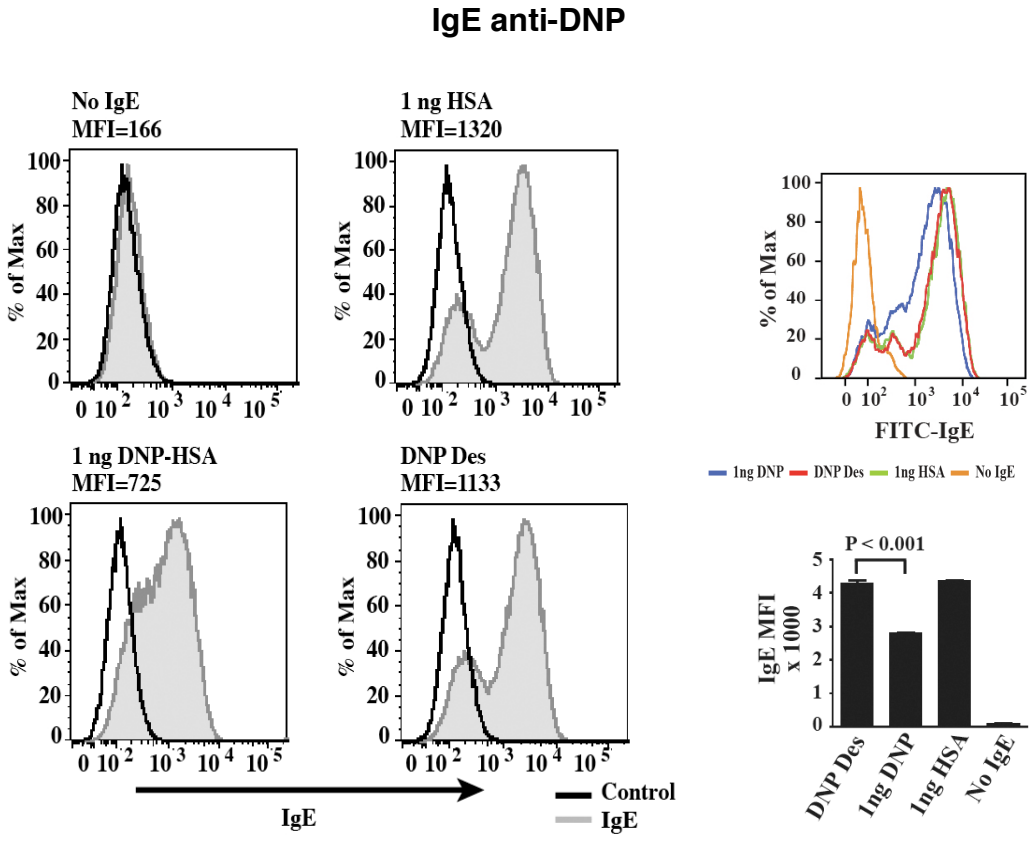


Figure 37. *Cells sensitized overnight with anti-DNP IgE or non-sensitized cells used as a control (No IgE), were treated as indicated. Representative histograms with IgE surface expression and mean fluorescence intensities are shown. Data are representative of four independent experiments.*

Surface expression levels of FcεRIα and IgE in desensitized cells were similar to those of cells challenged with 1 ng HSA and significantly higher than in activated cells (***Figure 37*** and ***Figure 38***), indicating the impairment of internalization of IgE and FcεRIα.

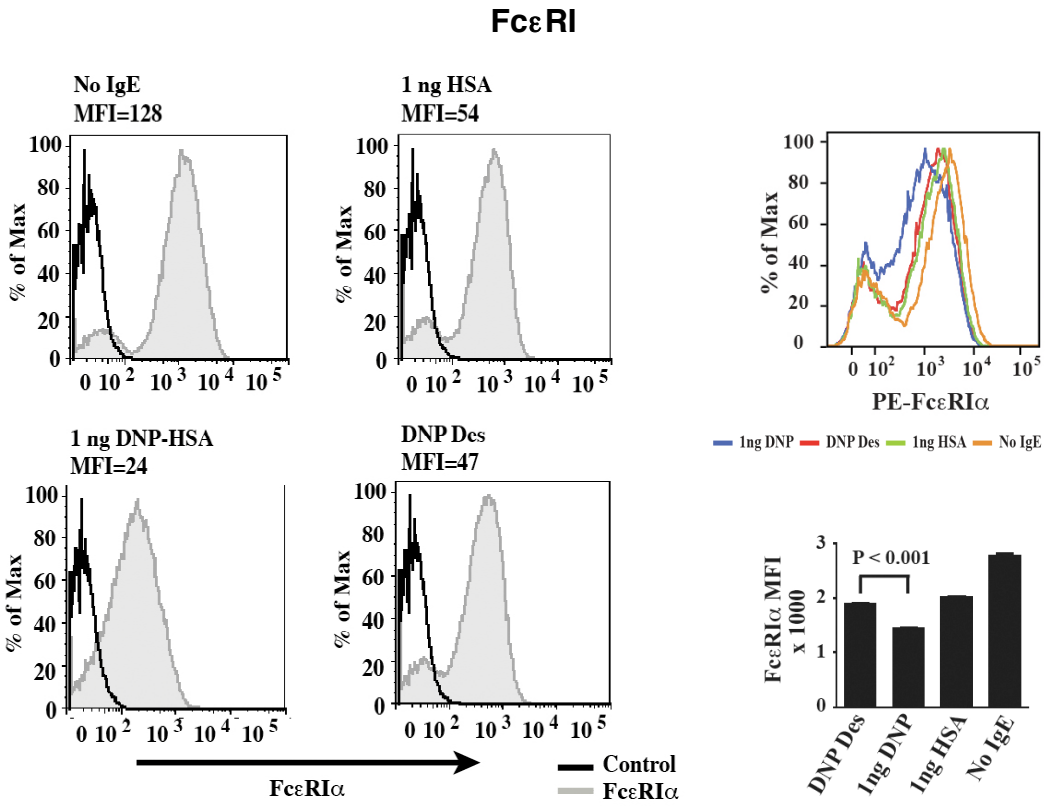


Figure 38. *Cells sensitized overnight with anti-DNP IgE or non-sensitized cells used as a control (No IgE), were treated as indicated. Representative histograms with FcεRIα surface expression and mean fluorescence intensities are shown. Data are representative of four independent experiments.*

12.2 Confocal images for OVA antigen internalization

To assess the fate of the desensitizing antigen, we used Alexa Fluor 488-conjugated OVA for activation and for desensitization.

Due to the low amount of OVA antigen used in our system and the impossibility of using such amount to obtain successful confocal images, the protocol was adapted to 50 ng OVA in spite of 10 ng OVA. This adapted protocol is specified in ***Table 7***.

Steps	Time (min)	Volume (μl) OVA	Concentration (pg/μl) OVA	Dose (pg) OVA
1	0	1	50	50
2	10	1	250	250
3	20	1	250	250
4	30	1	500	500
5	40	1	500	500
6	50	2	500	1000
7	60	2	1000	2000
8	70	4	1000	4000
9	80	8	1000	8000
10	90	16	1000	16000
11	100	17.5	1000	17500
11 steps	100 min	54.5 μl*		50 ng

*added to 50 μL of cells

Table 7. *Adapted protocol for rapid desensitization to 50 ng OVA*

We followed OVA antigen localization in activated and desensitized cells (***Figure 39***). A cross-sectional view of the intracellular compartment revealed that cells challenged with 50 ng of fluorescently labeled OVA showed large internalized aggregates, as confirmed by other researchers (Kambayashi T et al. (1)).

In contrast, OVA-desensitized cells showed fewer and smaller fluorescent aggregates, and their visual appearance was similar to that of cells challenged at 4°C, in which crosslinked receptors were not internalized and appeared with small aggregates bound to the membrane.

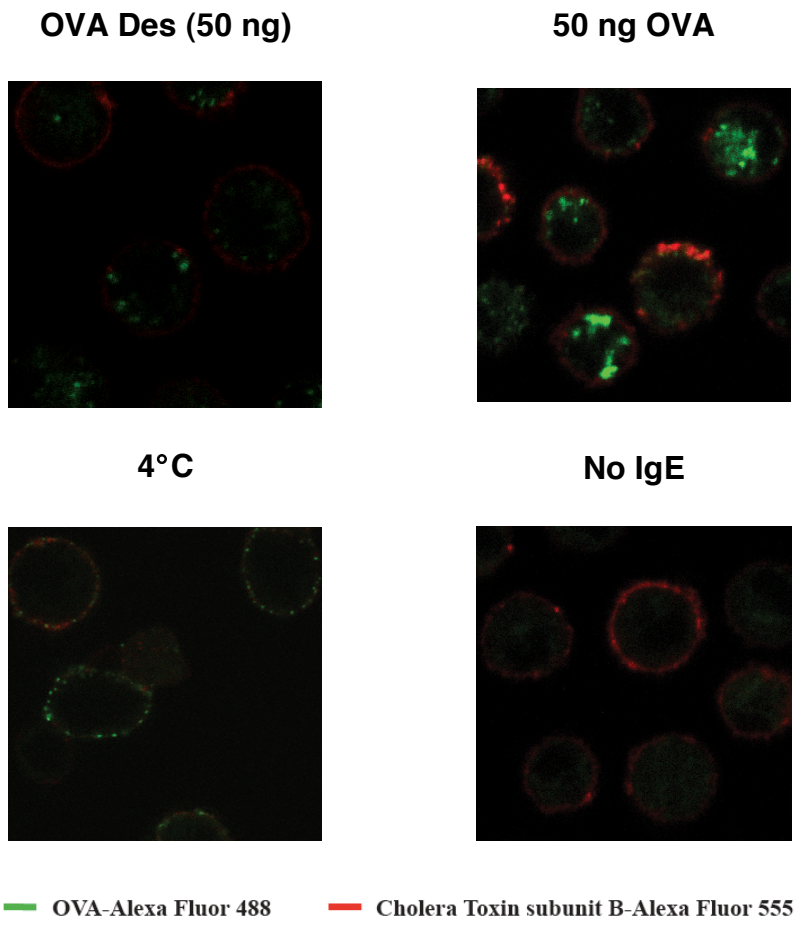


Figure 39. *Anti-OVA IgE sensitized cells or non-sensitized cells (No IgE) were treated as indicated and visualized by confocal microscopy. Cholera Toxin subunit B-Alexa Fluor 555 (red), OVA-Alexa Fluor 488 (green). Fields were obtained from one experiment and are representative of four independent experiments. The scale bar is 8.5μm and the original magnification is 630 x.*

13. Availability of free IgE receptors after desensitization

Since most of the IgE/FcεRI complexes remained on the cell surface, we sought to determine whether anti-IgE could crosslink free IgE on desensitized cells. DNP-desensitized cells released β-hexosaminidase when treated with anti-IgE (***Figure 40***), indicating that unbound IgE was available for cross-linking and remained accessible.

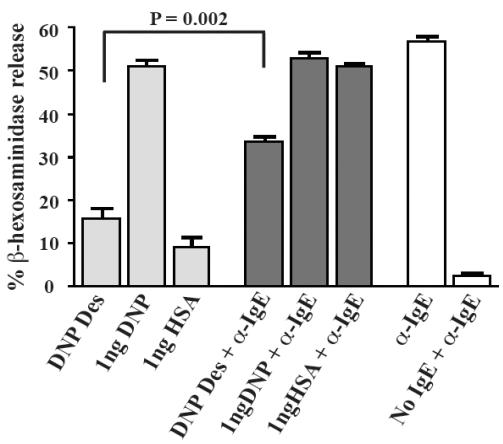


Figure 40. *β-hexosaminidase release from sensitized BMMCs desensitized, challenged with DNP-HSA or HSA, with (black bars) or without (grey bars) further crosslinking with anti-IgE Ab. Controls (white bars): sensitized and non sensitized cells crosslinked with anti-IgE Ab. Data are expressed as mean ± SEM of three independent experiments.*

14. Specificity of rapid desensitization

Since desensitized cells were hypo-responsive to further triggering doses of the same antigen, we studied the response to a second triggering antigen. Cells sensitized with anti-DNP IgE and anti-OVA IgE, were desensitized to OVA or to DNP and then challenged with triggering doses of DNP-HSA or OVA, respectively. We analyzed specificity from thre different points of view: degranulation (β-hexosaminidase release), calcium mobilization and confocal images with fluorescently labeled OVA and DNP antigens.

14.1 β-hexosaminidase release assay

As seen by β-hexosaminidase release assay, cells desensitized to OVA responded to a triggering dose of 1 ng DNP-HSA, and cells desensitized to DNP responded to a triggering dose of 10 ng OVA (***Figure 41***).

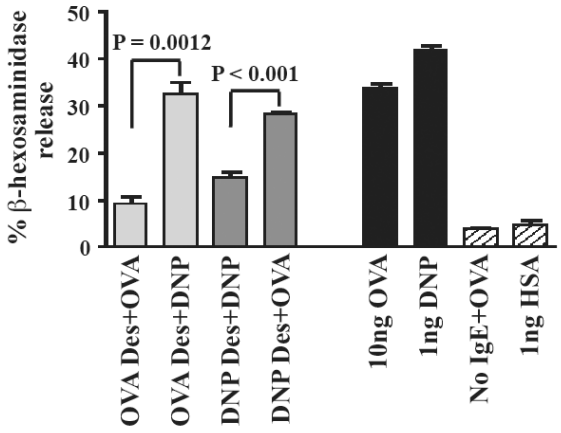


Figure 41. *β-hexosaminidase release from BMMCs in response to the indicated treatments. Cells were sensitized with both anti-OVA IgE and anti-DNP IgE and then desensitized to OVA (OVA Des) or to DNP (DNP Des) and then challenged with OVA and DNP-HSA (light grey) or viceversa (dark grey). OVA and DNP-HSA activation (black bars). Data are expressed as mean ± SEM of three independent experiments.*

These results indicated that mediators were not depleted after desensitization to one antigen and that desensitization disabled the specific response only to the desensitizing antigen.

14.2 Calcium mobilization assay

We then analyzed the specificity of the calcium responses. Cells desensitized to OVA had impaired calcium influx when triggered with 10 ng OVA, but the influx was restored by a triggering dose of 1 ng DNP-HSA (***Figure 42***, red line), indicating that the calcium response was compartmentalized by specific antigen.

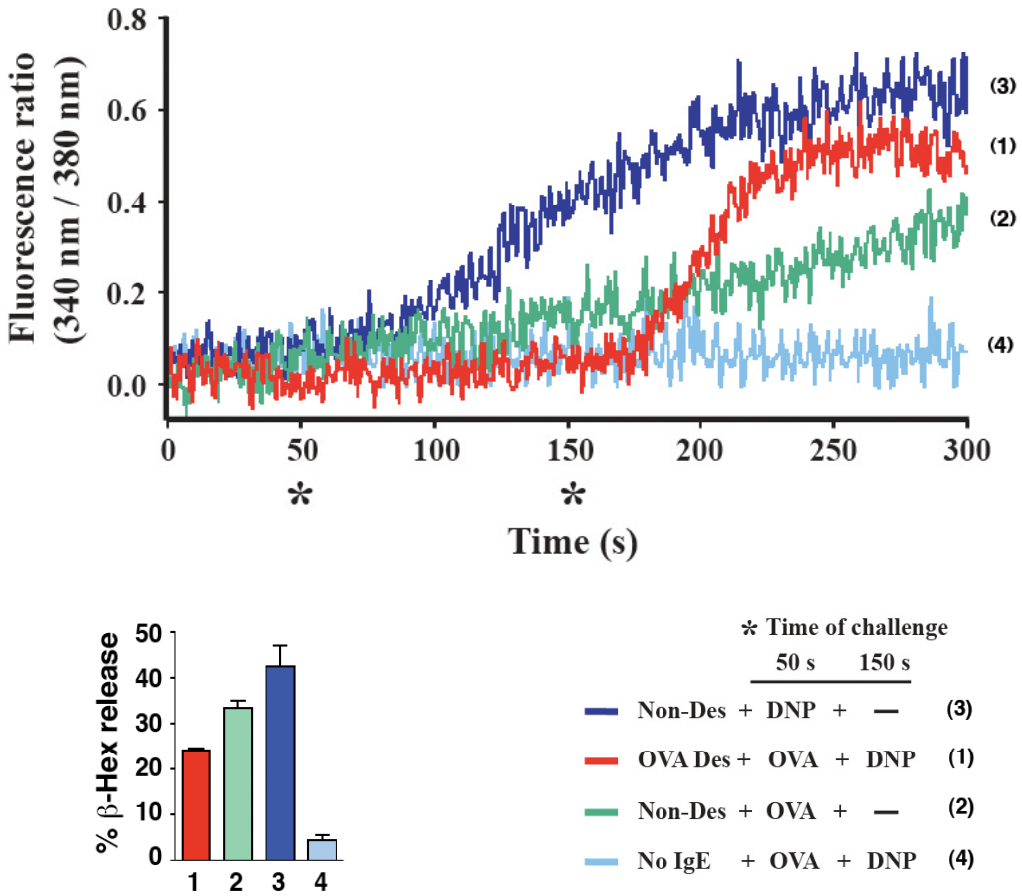


Figure 42. *10^6 mBMMCs were sensitized with both anti-DNP IgE and anti-OVA IgE and non-sensitized cells were used as negative control for OVA activation (No IgE). % β-hexosaminidase release and calcium flux of cells in response to the indicated treatments. Data are representative of three independent experiments.*

14.3 Confocal images of OVA and DNP antigens internalization

We then analyzed specificity using confocal microscopy (***Figure 43***). OVA-desensitized cells showed low internalization of labeled OVA antigen (green) as compared to the larger aggregates seen in OVA-activated cells. When OVA-desensitized cells were challenged with DNP-HSA (purple), the amount of internalization was comparable to that of DNP-HSA activated cells, indicating that desensitization left unaffected the specific mechanisms of cell activation and receptor internalization.

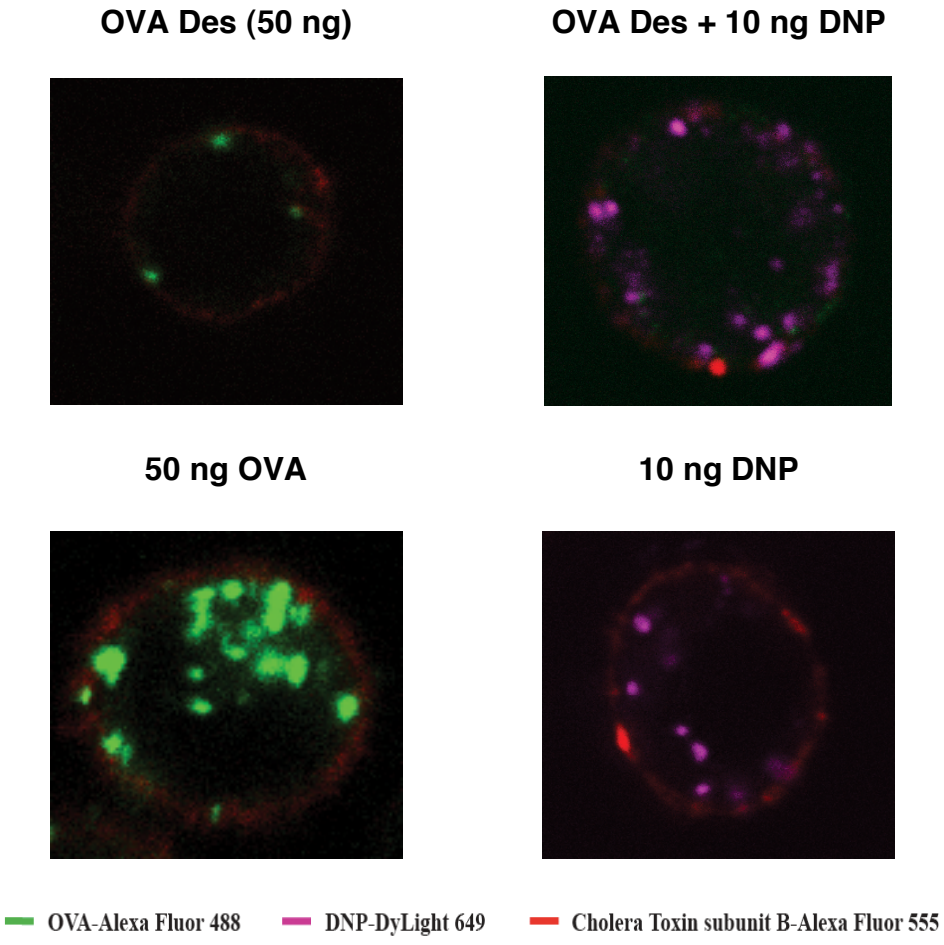


Figure 43. *Confocal microscopy of cells treated as indicated. Cholera Toxin subunit B-Alexa Fluor 555 (red), OVA-Alexa Fluor 488 (green) and DNP-DyLight 649 (purple). Fields were obtained from one experiment and are representative of three independent experiments. The scale bar is 3μm and the original magnification is 630 x.*

15. Actin polimerization during desensitization

It has been hypothesized that actin microfilaments might be involved in the down-regulation of the degranulation response. The purpose of this experiment was to investigate if actin polimerization affected the desensitization process.

It was determined that there is a good correlation between inhibition of actin polymerization and increases in tyrosine kinase activity and degranulation and that actin microfilaments appear to down-regulate the response by affecting the level of receptor tyrosine phosphorylation, thus affecting all the signaling pathways involved.

Cytochalasin D is an actin-modifying drug and a cell-permeant fungal toxin that binds to the barbed end of actin filaments, inhibiting both the association and dissociation of subunits, leading to stabilization of the actin filaments. Under normal circumstances, activation of mast cells with antigen leads to increased actin polymerization and this is inhibited by cytochalasin D pretreatment.

Desensitization to 1ng of DNP-HSA, activation with 1ng DNP-HSA or challenge with 1ng HSA, were done with or without a 15-minute pre-incubation with Cytochalasin D and degranulation was monitored by β-hexosaminidase release (***Figure 44***).

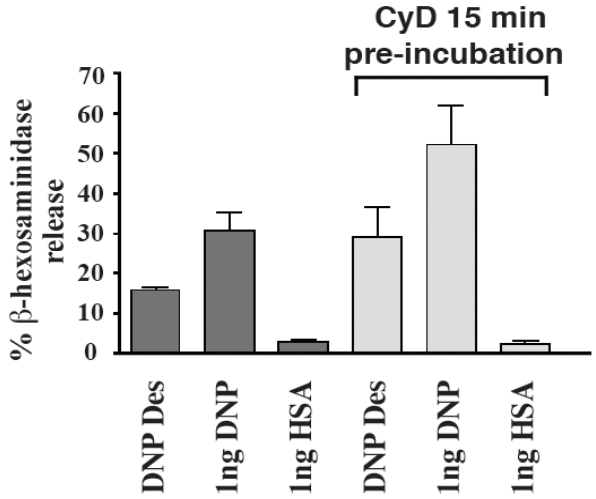


Figure 44. *β-hexosaminidase release from overnight sensitized RBL-2H3 cells that were desensitized, challenged with 1 ng DNP-HSA and with 1 ng HSA with or without pre-treatment with cytochalasin D. Data are expressed as mean ± SEM of three independent experiments.*

In accordance with a previous report in which cytochalasin D treatment of RBL-2H3 cells prevented antigen-stimulated actin polymerization and was accompanied by increasing degranulation (Frigeri L et al.), β-hexosaminidase release in activated cells pre-treated with cytochalasin D was enhanced 2-fold compared with activated untreated cells. This result was paralleled by desensitized cells, thus cytochalasin D treated RBL-2H3 cells were as successfully desensitized (55%) as the non-treated ones (52%).

DISCUSSION

Rapid drug desensitization procedures have emerged as critical components of treatment for allergic patients in need of first line therapy. These protocols have been refined to allow for safe delivery of chemotherapy drugs, antibiotics, monoclonals and other life saving medication in a very short time to sick patients (Castells et al. (3) / Lee CW et al. (1) / Lee CW et al. (2) / Legere HJ 3rd et al. / Brennan PJ et al.).

Due to their success and relatively safe outcomes, rapid desensitization protocols have been produced recently to prevent anaphylaxis in food allergic patients when exposed to food antigens (Mansfield L). Although these procedures are elective, they are aimed at improving the quality of life of millions of patients who can be accidentally exposed to foods that can trigger deadly reactions. The lack of severe anaphylactic reactions in clinical reports (Lee CW et al. (2) / Castells et al. (3)), including hundreds of drug desensitizations using a standardized 12-step protocol, illustrates a profound inhibition of acute and delayed in vivo mast cell activation responses.

Despite the clinical success, the mechanisms of rapid desensitization are not well understood and most of the molecular players have remained elusive. Our understanding of IgE desensitizations has been hampered by the lack of *in vitro* mast cell models providing quantitative and qualitative insight into the early and late activation responses. Multiple studies have been done with mast cells and basophils, but most of them in calcium-depleted conditions (non physiologic conditions) that could not be extrapolated to humans.

The present study describes a physiologic mouse mast cell model of rapid desensitization to antigens in which increasing doses of antigens added at fixed time intervals renders mast cells hypo-responsive. This *in vitro* study under physiologic calcium conditions provides proof of concept for the effectiveness, safety and specificity of human desensitizations.

The rapid (100 minutes) desensitization protocol presented here is based on previous data from Dr. Mariana Castells laboratory and others (Morales AR et al. / Shalit M et al.) in which it was shown that the dose escalation (2 or 10 fold) and the time between doses (5 or 30 min) were critical parameters in order to achieve desensitization. In the present protocol antigens are delivered at 10-min intervals in a cumulative fashion that provides an optimal dose-time relationship (***Table 5***). When starting at 1/1000 the target dose, the sequential delivery of increasing doses of antigen induced an almost complete inhibition of the activating responses seen in the dose-response curve. Each additional dose decreased responsiveness, and all 11 steps of the protocol were necessary for mast cells to become desensitized, since the greatest inhibition took place at the highest cumulative dose (***Figure 20***). This indicates that desensitization is a dynamic process in which each step provides a baseline platform for the next level of response reduction and that once desensitized, mast cells remain hyporesponsive to further antigen challenges (***Figure 21***). Increasing the target antigen dose for desensitization decreased the hyporesponsiveness (***Figure 22***), underscoring a critical relationship between IgE sensitization and the target

antigen dose for desensitization. Whether additional steps and/or time between steps might help achieve more hyporesponsiveness was not explored. In human desensitizations the level of IgE sensitization varies and is unknown for each patient and the target dose used for desensitization is empirical, which impacts its safety (Castells et al. (3) / Lee CW et al. (1) / Lee CW et al. (2) / Legere HJ 3rd et al. / Brennan PJ et al.).

Once mast cells are triggered by antigen, there is depletion of the ER stores leading to activation of plasma membrane calcium-release activated calcium (CRAC) channels that allow Ca^{2+} entry. Consistent with a lack of degranulation, entry of extracellular calcium was almost completely inhibited (***Figure 27***) in desensitized mast cells. The sequential delivery of low antigen doses during desensitization may provide continued low levels of calcium entry and induce conformational changes of CRAC and other calcium-related channels locking further calcium entry and blocking signal transduction. Because calcium entry is clearly specifically impaired in our model, since a second non-desensitizing antigen allowed restoration of calcium flux, membrane compartmentalization may be required to exclude signal transduction molecules around desensitized receptors. It is also possible that a threshold calcium signal to activate ER Ca^{2+} sensors cannot be reached during desensitization, preventing calcium influx and degranulation.

It has been shown that *in vitro* rapid desensitization of human mast cells induces decreased levels of signal-transducing molecules, such as syk, because of ubiquitinilation and degradation (Macglashan D et al. (2) / Odom S et al.) and that naturally occurring syk-deficient basophils are unresponsive to drug antigens (Gomez G et al.), indicating that syk is critical for activation and for desensitization (Kepley CL. (2)). In our hands, detection of Syk phosphorylation with desensitization was unsuccessful, possibly due to rapid phosphorylation-dephosphorylation and/or degradation of Syk during the desensitization process. However, since LAT, that is phosphorylated by Syk, serves as a docking site for further events leading to degranulation (Kraft S et al.) and LAT-deficient BMMC have impaired calcium mobilization and granule mediator release (Saitoh S et al. (1)), we established the fate of LAT and p38 MAP kinase. Both molecules phosphorylation were impaired in desensitized cells and consequently TNF-α and IL-6 production were diminished.

STAT6, which is responsible for the transcription of IL-4 and IL-13, has been also involved in rapid desensitizations. STAT-6-deficient mast cells are capable of releasing mediators during the early phase of IgE cell activation but cannot release late cytokines, such as tumor necrosis factor TNF-α and IL-6 (Malaviya R et al.), and cannot be desensitized to DNP antigen (Morales AR et al.). STAT-6 phosphorylation was impaired in desensitized cells and it is possible that STAT6 activity is required for desensitization, via a pathway different from the one leading to the acute and late activating responses.

Our system is limited by the fact that BMMC are cultured in IL-3, which may affect cytokine production (Gonzalez-Espinosa C et al.). Nonetheless, this may have an important correlate in human desensitizations since our group has not observed delayed reactions in desensitized patients, confirming that the inhibition of mast cell activation during desensitization prevented later hypersensitivity reactions (Lee CW et al. (2) / Castells MC et al. (3)).

In humans, temporary tolerization is achieved in hours and can be maintained if drug antigens are administered at regular intervals, depending on pharmacokinetic parameters (Castells M (1)). Maintenance of hyporesponsiveness in desensitized cells was not sustained by the presence of an excess of soluble antigen since washed cells remained desensitized. It is possible that bound antigen is equilibrated in desensitized cells. Earlier studies (Shalit M et al. / Rubinchik E et al.) suggested that the hypo-responsiveness induced by desensitization was due to internalization of antigen/IgE/FcεRI complexes and that the lack of available IgE renders the cells refractory to further stimulation. In contrast, we show here that, unlike in activation, during desensitization internalization of IgE and FcεRI is impaired (***Figure 37*** and ***Figure 38***) and that desensitized cells can be triggered by anti-IgE, since unbound IgEs remain accessible and are available for cross-linking (***Figure 40***). Saturating doses of IgE in a co-culture system and the use of higher antigen doses (Shalit M et al.) promote internalization while low doses may redistribute antigen-bound receptor at the membrane level. Moreover, others have shown that low doses of antigen can crosslink IgE receptors, thus remaining mobile on the cell surface whereas high doses of antigen immobilize IgE-crosslinked receptors and induce degranulation (Andrews NL et al.). In that study mobile receptors bound to low dose of antigen had a threshold for activation responses. It is possible that during desensitization the low incremental additions of antigen promote receptor mobility and lack of internalization. Other studies suggested that activation of FcεRI induced by low antigen concentrations results in nuclear signals in the absence of degranulation (Grodzki AC et al.) but our results show that with desensitization, there is no phosphorylation of p38-MAPK (***Figure 32***) no IL-6 and no pre-formed or newly generated TNF-α (***Figure 25*** and ***Figure 33***).

The substrate component of specific desensitization remains unsolved, and it is of particular importance in desensitized patients since mast cell reactivity is maintained for non-desensitizing antigens. Previous studies in calcium-depleted conditions have shown that cells desensitized to one antigen could not be triggered with a non-desensitizing second antigen (MacGlashan DJr et al. (3)), possibly through a disabling mechanism involving syk ubiquitination (MacGlashan D et al. (2)). Due to the amount of IgE sensitization and low antigen doses used in our model, we could not detect syk phosphorylation but it is also possible that low amounts of antigen cannot trigger its phosphorylation.

Confocal microscopy studies gave us the opportunity to directly look into the antigen localization after desensitization. Our findings indicate that the mast cell activating machinery was intact for a non-desensitizing antigen action, since no mediator depletion occurred with desensitization. Calcium flux was restored in desensitized cells when challenged with a non-desensitizing antigen (***Figure 42***), and microscopic images confirmed that rapid desensitization is antigen specific (***Figure 43***) and does not induce anergy (MacGlashan DJr et al. 2008).

Mast cell activation requires coordinated events in order to respond to an allergen and may differ depending on the type and strength of a stimulus. These events begin with the allergen-dependent aggregation of the IgE antibody-occupied high affinity receptor for IgE (FcεRI) and are propagated inside the cell through a sophisticated network of signaling molecules. While we do not know the exact mechanism that could explain the inhibition of receptor internalization during desensitization, it is possible that the mobility of antigen/IgE/FcεRI complexes and membrane re-arrangement could prevent their internalization, as shown by others with low doses of multivalent antigen (Andrews NL et al.). In addition, receptors engaged with low doses of antigen could be segregated into different compartments (Simons K et al.), preventing access to phosphorylating molecules. Inhibitory phosphatases such as SHP-1 may not be excluded from those compartments, thus preventing phosphorylation of key molecules required for signal transduction. A time course study of SHP-1 phosphorylation in RBL-2H3 cells (Ozawa T et al.) has shown a peak at 1 min of FcεRI crosslinking and a gradually decline within 10 min. Our initial results indicated a lack of phosphorylation at 100 min. Further studies are planned to look for phosphorylation of SHP-1 and other ITIM-bearing molecules (Lu-Kuo JM et al. / Ozawa T et al. / Castells MC et al. (2)) at each step of the desensitization protocol since it may be transient.

Because our study indicates a strong inhibition of early signal transduction, it is possible that inhibitory molecules, such as $LILRB_4$ that become phosphorylated upon IgE crosslinking could dephosphorylate the β and the γ chains of the FcεRI upon antigen engagement. The molecular mechanism by which gp49B1 inhibits the FcεRI-induced mast cell activation seems to involve SHP-1 rather than SHP-2. As shown in our study, inhibition of internalization prevents calcium entry, which would abrogate the acute and late phase activation. Indeed, co-clustering of gp49B1 with FcεRI on mast cells inhibits the induced secretion of stored proinflammatory mediators as well as of newly generated lipid mediators (Lu-Kuo JM et al. / Castells MC et al. (2) / Katz HR et al. (2)).
Another molecule that we have not explored is the inositol phosphatase SHIP that has been reported to bind directly to the FcεRI β chain *in vitro* and regulates FcεRI signal transduction independently from FcγRIIB (Osborne MA et al. / Kimura T et al.). It has also been shown that FcεRI-induced calcium mobilization and MAPK activation are enhanced in BMMC derived from SHIP-deficient mice compared to wild-type mice (Huber M et al.). Thus it is also possible that SHIP could allow for desensitization.

Whether SHIP, $LILRB_4$ or other ITIM-containing mast cell inhibitory receptors participate in the gradual re-arrangement of the antigen-crosslinked receptor preventing its internalization needs to be explored.

Rat basophilic leukemia (RBL 2H3) cell line has been commonly used as a histamine-releasing cell line and has been proved to be a suitable model to examine signal transduction events in FcεRI mediated mast cell activation. We decided to use this cell line due to their adhesive characteristics for further confocal microscopy experiments but also for actin polimerization studies. It has been hypothesized that actin microfilaments might be involved in the down-regulation of the degranulation response at the level of receptor phosphorylation (Frigeri L et al. / Benhamou M et al.) and that actin assembly and reorganization plays an important role in FcεRI-induced mast cell degranulation and calcium signaling (Oka T et al. / Wu M et al.). This is what drives us to investigate if desensitization could be affected by actin polimerization. Our results, accordingly with those previously cited, determined that there is a good correlation between inhibition of actin polymerization and increases in degranulation for activation as well as for desensitization (***Figure 44***). Thus, actin microfilaments could down-regulate the response by affecting the level of receptor tyrosine phosphorylation and uncoupling Lyn from the cross-linked receptor, but since our results showed that the antigen/IgE/FcεRI complexes do not internalize with desensitization, further receptor phosphorylation could not be relevant.

The data presented here provides a unique *in vitro* model of rapid IgE desensitization, under physiologic calcium conditions, that is effective and reproducible and sheds some light on the mechanisms underlying downregulation of mast cell responses.

This data shows how an optimal antigen dose-time relationship leads to almost complete abrogation of early- and late-phase activation events and establishes the first *in vitro* model of antigen-specific desensitization, disabling the specific response to one antigen but keeping the cell machinery unaffected, unlike non-specific desensitization. Furthermore, there is a clear demonstration that low doses of antigen sequentially added are inhibitory for mast cell activation in the presence of calcium, and induce no depletion of mediators. Most importantly, this data demonstrates that specific rapid desensitization inhibits the internalization of the antigen/IgE/FcεRI complexes.

We speculate that the cellular cytoskeleton plays an important role in the desensitization process, such that antigen/IgE/FcεRI complexes possibly remain mobile on the surface of the cell during rapid desensitization allowing their re-arrangement. The plasma membrane may redistribute selectively with the desensitized receptor clusters allowing compartmentalization and possibly segregation of activating molecules therefore impairing downstream signal transduction. Additionally, inhibitory molecules may become associated with desensitized receptor clusters preventing cellular activation. A simplified cartoon in ***Figure 45*** compares the outcomes of mBMMC activation and rapid

desensitization and presents our hypothesis explaining how desensitization affects antigen/IgE/FcεRI complexes re-arrangement at the cell membrane.

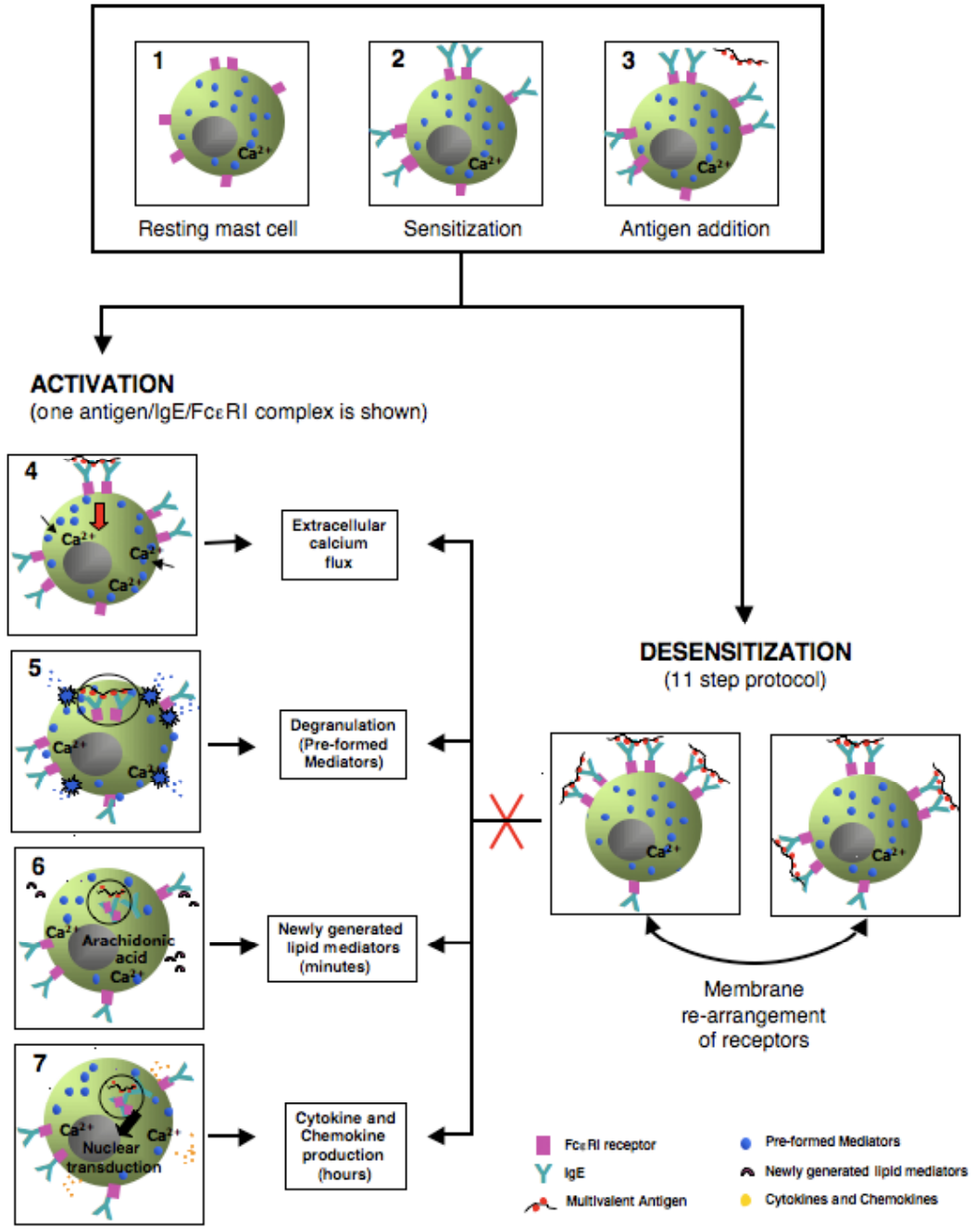


Figure 45. *Simplified cartoon comparing activation and desensitization outcomes as well as a possible explanation of how rapid desensitization works and the re-arrangement of the FcεRI receptors at the cell membrane.*

CONCLUSIONS

In this PhD thesis,

1. we established the first *in vitro* model of m-BMMC IgE rapid desensitization
   - that provides an optimal relationship between antigen dose and time between doses
   - that is effective and reproducible, leading to almost complete abrogation (50-80%) of mast cell activation events
   - that is versatile (can be used for various target doses, with several antigens and with different cell types)

2. we demonstrated that m-BMMC IgE rapid desensitization
   - inhibits acute and late phase mast cell responses
   - is an antigen specific process that disables the specific response to one antigen but keeps the cell machinery unaffected, unlike non-specific desensitization
   - can be maintained, in the presence or absence of antigen, up to 6 h
   - impairs internalization of the FcεRI /IgE/antigen complexes

CONCLUSIONES

En esta tesis,

1. se ha establecido el primer modelo *in vitro* de desensibilización rápida por IgE de mastocito derivado de médula ósea de ratón (m-BMMC)
   - que proporciona una relación óptima dosis de antígeno-tiempo entre dosis
   - que es efectivo y reproducible, llegando hasta casi la completa desaparición (50-80%) de la activatión del mastocito
   - que es versátil (puede ser usado con varias dosis objetivo, con diversos antígenos y con diferentes tipos de células)

2. se ha demostrado que la desensibilización rápida por IgE de m-BMMC
   - inhibe la respuesta inmediata y la respuesta tardía del mastocito
   - es un proceso específico para antígeno que discapacita la respuesta específica a un antígeno pero mantiene la maquinaria celular intacta, no como en la desensibilización no específica
   - se puede mantener hasta 6 h en presencia o ausencia de antígeno
   - afecta la internalización del complejo antígeno /IgE/ FcεRI

PERSPECTIVES

Rapid drug desensitizations are currently emerging as highly effective and safe procedures for the administration of important medications while minimizing or entirely circumventing hypersensitivity reactions in sensitized patients.

Strategies aimed at dilucidating the full mechanism underlying rapid desensitization may constitute an important tool for a better understanding of the process and for the development of improved and safer protocols for drug and food desensitizations.

We therefore anticipate that future research will also focus on using our *in vitro* model as the first step for using patient serum and establishing the minimal dose and concentration for the initial desensitization steps and the creation of individualized desensitization protocols.

PUBLICATIONS

Article

Sancho-Serra Mdel C, Simarro M, Castells M. *Rapid IgE desensitization is antigen specific and impairs early and late mast cell responses targeting FcεRI internalization.*
European Journal of Immunology 2011 Apr;41(4):1004-13

Review

Liu A, Fanning L, Chong H, Fernandez J, Sloane D, Sancho-Serra M, Castells M. *Desensitization regimens for drug allergy: state of the art in the 21st century.*
Clinical & Experimental Allergy 2011 Aug 23. doi: 10.1111. Review

Book Chapter (in press)

Karger Book Series: Chemical Immunology and Allergy 2011
Book title**: Adverse Cutaneous Drug Eruptions**
Chapter: *Desensitization for hypersensitivity reactions to medications*
Authors: Sancho M., Breslow R., Sloane D., and Castells M.

Abstract

Rapid IgE-antigen desensitization of mouse bone marrow derived mast cells inhibits degranulation, STAT6 phosphorylation and calcium release in an antigen-specific manner.
Collegium Internationale Allergologicum
27th Symposium in Curaçao, 2008

Poster

Rapid IgE desensitization of mast cells impairs acute and late-phase activation responses
Rheumatology, Immunology and Allergy Division Workshop 2009

Oral presentation

Rapid IgE Desensitization of Mast Cells Impairs Acute and Late-phase Activation Responses and Antigen Internalization
American Academy of Allergy, Asthma & Immunology
New Orleans 2010 Annual Meeting

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ANNEX

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Rapid IgE desensitization is antigen specific and impairs early and late mast cell responses targeting FcεRI internalization

Maria del Carmen Sancho-Serra[1], Maria Simarro[2] and Mariana Castells[1]

[1] Division of Rheumatology, Immunology and Allergy, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA
[2] Unidad de Investigacion, Hospital Clinico Universitario de Valladolid, Valladolid, Spain

Rapid IgE desensitization provides temporary tolerization for patients who have presented severe hypersensitivity reactions to food and drugs, protecting them from anaphylaxis, but the underlying mechanisms are still incompletely understood. Thus, here we develop an effective and reproducible in vitro model of rapid IgE desensitization for mouse BM-derived mast cells (BMMCs) under physiologic calcium conditions, and we characterize its antigen specificity and primary events. BMMCs were challenged with DNP-human serum albumin conjugated (DNP-HSA) and/or OVA antigens, delivered either as a single dose (activation) or as increasing sequential doses (desensitization). Compared to activated cells, desensitized BMMCs had impaired degranulation, calcium flux, secretion of arachidonic acid products, early and late TNF-α production, IL-6 production, and phosphorylation of STAT6 and p38 mitogen-activated protein kinase (p38 MAPK). OVA-desensitized cells responded to DNP and DNP-desensitized cells responded to OVA, proving specificity. Internalization of specific antigen, IgE and high-affinity receptor for IgE (FcεRI) were impaired in desensitized BMMCs. Our results demonstrate that rapid IgE desensitization is antigen specific and inhibits early and late mast cell activation responses and internalization of the antigen/IgE/FcεRI complexes.

Keywords: Desensitization · DNP/OVA · FcεRI · IgE · Mast cells

Introduction

Exposure of IgE-sensitized patients to medication or food allergens can cause the sudden systemic release of inflammatory mediators from activated mast cells, leading to anaphylaxis [1, 2]. Avoidance may be difficult for food-sensitized patients due to cross-reactive food allergens. For medication-sensitized patients, avoidance may lead to significant morbidity and mortality if treatment for cancer or severe infection becomes necessary, and may decrease the quality of life among patients with chronic inflammatory diseases sensitized to monoclonal antibodies. Desensitization protocols have been developed to help deliver full therapeutic doses of drug allergens, in an incremental, stepwise fashion without eliciting life-threatening symptoms [3–5]. More recently, food desensitization protocols have been generated to protect children and adults from accidental exposures to allergenic foods [6, 7]. Most IgE-sensitized patients present a positive skin test to the offending food or medication, indicating that mast cells and IgE are the main targets of these reactions. After rapid desensitization, specific skin test reactivity is abolished, implying a complete inhibition of the mechanisms that induce mast cell activation [8].

Correspondence: Dr. Mariana Castells
e-mail: mcastells@partners.org

Mast cells are activated by antigen crosslinking of IgE-bound high-affinity receptor for IgE (FcεRI) receptors, and aggregation of these receptors results in rapid phosphorylation of tyrosine residues in the ITAMs of β and γ chains by lyn kinase, which leads to recruitment and activation of spleen tyrosine kinase (syk) and fyn. Both fyn and syk phosphorylate downstream targets, leading to calcium mobilization, degranulation, arachidonic acid metabolization, and cytokine and chemokine gene transcription [9, 10]. As opposed to activation, desensitization is a process in which mast cells are rendered hypo-responsive to an activating challenge, either by exposure to low antigen doses in calcium-depleted conditions [11] or by exposure to incremental doses of antigen, in the presence of calcium [12, 13]. Calcium-depleted conditions cannot be applied to human desensitizations, and few studies have addressed physiological desensitizations, since events occurring in the absence of extracellular calcium may not reflect the same pathways as those occurring in the presence of calcium [14]. Internalization of FcεRI through progressive crosslinking at low levels of antigen has been postulated as the likely mechanism for cell-surface depletion of IgE and cellular unresponsiveness to specific activating doses of allergen [12]. Depletion of molecular targets of activation such as syk has been shown in prolonged antigen desensitization, indicating a universal rather than specific desensitization [15].

Based on our previous study [16], we report here a model of mouse BM-derived mast cell (BMMCs) specific rapid desensitization to DNP and OVA antigens in the presence of physiologic levels of calcium. Increasing doses of antigen delivered at fixed time intervals induced a highly specific and prolonged hypo-responsiveness to triggering doses of the desensitizing antigen. Mast cells desensitized to DNP or OVA demonstrated almost complete inhibition of β-hexosaminidase and pre-formed TNF-α release, calcium flux and arachidonic acid metabolization. They did not release significant amounts of newly generated IL-6 or TNF-α and failed to phosphorylate STAT6 and p38 MAPK. When sensitized to both DNP and OVA antigens, DNP-desensitized cells responded fully to OVA and vice versa. Most importantly, specific rapid desensitization targeted the internalization of antigen/IgE/FcεRI complexes since antigen-specific IgE bound to the α chain of the FcεRI remained at the membrane level. This model may provide support for the specificity and effectiveness of human desensitizations.

Results

Inhibition of BMMC responses to sequential versus single-dose DNP antigen delivery

In order to compare single-dose antigen delivery (activation) with sequential cumulative doses (rapid desensitization), we first assessed the dose response curve to DNP-human serum albumin conjugated (DNP-HSA) antigen, by β-hexosaminidase release, with cells sensitized with anti-DNP IgE (see Fig. 1A). DNP-HSA doses of 1, 5 and 10 pg (DNP in figure) were non-activating as single doses, since the percentage of β-hexosaminidase release was comparable to that of the control (1 ng HSA). Cells challenged with higher doses of antigen (>10 pg DNP-HSA) delivered as single doses achieved significant β-hexosaminidase release. The black bar in Fig. 1A (1 ng DNP) represents the optimal triggering dose of 1 ng DNP-HSA used as target dose for rapid desensitization to 1 ng of DNP-HSA (DNP Des). The release obtained with single-dose additions in Fig. 1A was compared to that obtained with doses added sequentially, following every step of the desensitization protocol (see Fig. 1B, white bars). White bars represent β-hexosaminidase release at each particular point in the cumulative sequence of antigen additions. A maximum of 10% β-hexosaminidase release was achieved at all points in the sequence, showing that the desensitization process did not induce a slow release of mediators.

To determine whether there was a threshold dose that initiated hypo-responsiveness, replicate samples were used, and at each particular point in the sequence of antigen additions, cells were also challenged with a triggering dose of 1 ng DNP-HSA (see Fig. 1B, gray bars). Response to the triggering dose declined with increasing number of sequential doses. The greatest hypo-responsiveness was achieved with the highest number of sequential additions (11, in Fig. 1B), indicating that hypo-responsiveness was not stabilized until the end of the desensitization protocol.

To test whether cells' hypo-responsiveness achieved with rapid desensitization to 1 ng DNP-HSA could be overcome with higher challenging doses, we analyzed the response of desensitized cells to activating doses of 1, 2, 3, 4 and 5 ng of DNP-HSA. Up to five-fold increase in challenging dose did not reverse desensitization (see Fig. 1C).

The protocol was effective when increasing the target dose to 5 and 10 ng, with the same number of steps, time between steps and starting dose (1/1000 the target dose), but less inhibition of β-hexosaminidase release was observed (see Fig. 1D). Cells desensitized to 1 ng DNP-HSA showed a 75% inhibition whereas cells desensitized to 5 and 10 ng DNP-HSA had a 65 and 41% inhibition of β-hexosaminidase release, respectively.

BMMC rapid desensitization impairs early and late phase cell responses

BMMCs sensitized with anti-DNP IgE or anti-OVA IgE were rapid-desensitized as per the protocol presented in Table 1. In both DNP and OVA systems, we measured the release of β-hexosaminidase when antigen was delivered as a single dose (1 ng DNP-HSA/10 ng OVA, black bars in Fig. 2A) or when antigen was delivered following the rapid desensitization protocol (white bars in Fig. 2A). Cells desensitized to 1 ng DNP-HSA and 10 ng OVA showed a 78 and 71% inhibition of β-hexosaminidase, respectively.

Exocytosis of pre-formed mediators from granules cannot occur without external calcium entry. During mast cell activation, the release of calcium from the endoplasmic reticulum provides

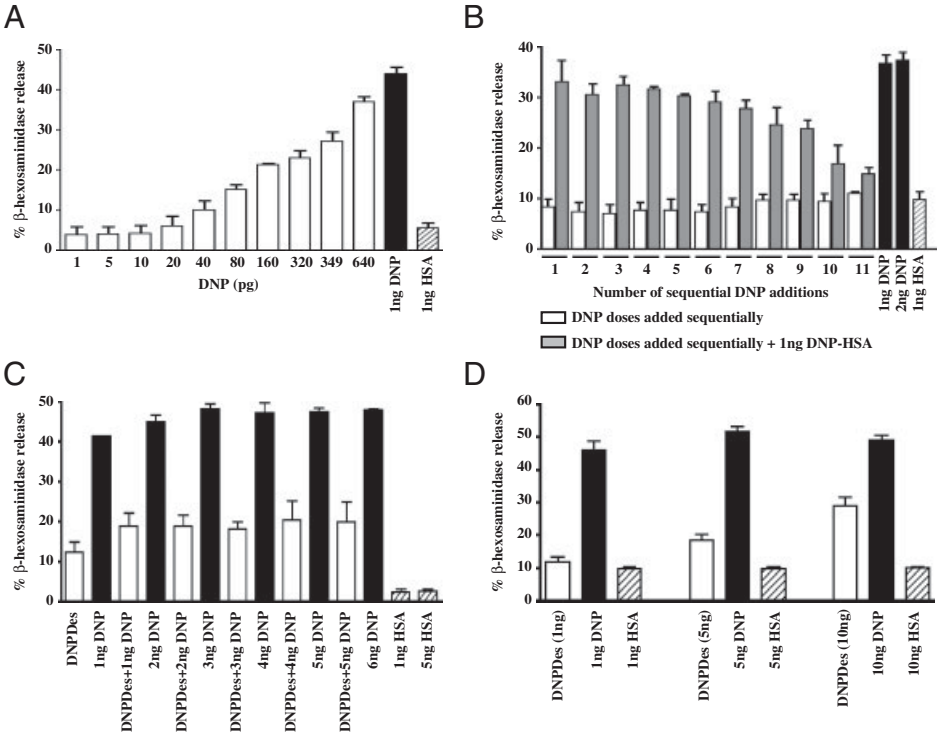

Figure 1. Inhibition of BMMC responses to sequential versus single-dose DNP antigen delivery. Percentage of β-hexosaminidase release assay in cells sensitized overnight with anti-DNP IgE. (A) Dose response to DNP-HSA. (B) DNP-HSA doses sequentially added. White bars show accumulation of β-hexosaminidase at each particular point in the sequence of DNP-HSA additions (DNP in the graph) as per protocol in Table 1. Grey bars show replicate samples in which 1 ng DNP-HSA was added 10 min after the last DNP-HSA addition in the sequence of DNP-HSA additions as per protocol in Table 1. (C) Responsiveness of desensitized BMMCs to different DNP-HSA challenge doses. (D) Rapid desensitizations to 1, 5 and 10 ng of DNP-HSA. All data are expressed as mean+SEM of three independent experiments.

Table 1. Rapid desensitization protocol[a)]

Steps	Time (min)	Volume (μL)	Concentration (pg/μL)		Dose (pg)	
		DNP-HSA/OVA	DNP-HSA	OVA	DNP-HSA	OVA
1	0	1	1	10	1	10
2	10	1	5	50	5	50
3	20	1	5	50	5	50
4	30	1	10	100	10	100
5	40	1	10	100	10	100
6	50	2	10	100	20	200
7	60	2	20	200	40	400
8	70	4	20	200	80	800
9	80	8	20	200	160	1600
10	90	16	20	200	320	3200
11	100	17.5	20	200	350	3500
11 Steps	100 min	54.5 μL[b)]			1 ng	10 ng

[a)] Eleven incremental doses of antigen DNP-HSA or OVA were delivered to BMMCs at fixed time intervals until the target dose (1 ng DNP or 10 ng OVA) was reached.
[b)] Added to 50 μL of cells.

the signal for calcium-release-activated calcium (CRAC) channels to open, allowing extracellular calcium flux [17]. We compared changes in fluorescence ratios when a triggering dose of 1 ng DNP-HSA was added to non-desensitized cells, to desensitized cells and to cells that had not been sensitized with anti-DNP IgE. DNP-desensitized cells showed 90% inhibition of calcium mobilization (see Fig. 2B), indicating that calcium-dependent events are impaired during desensitization.

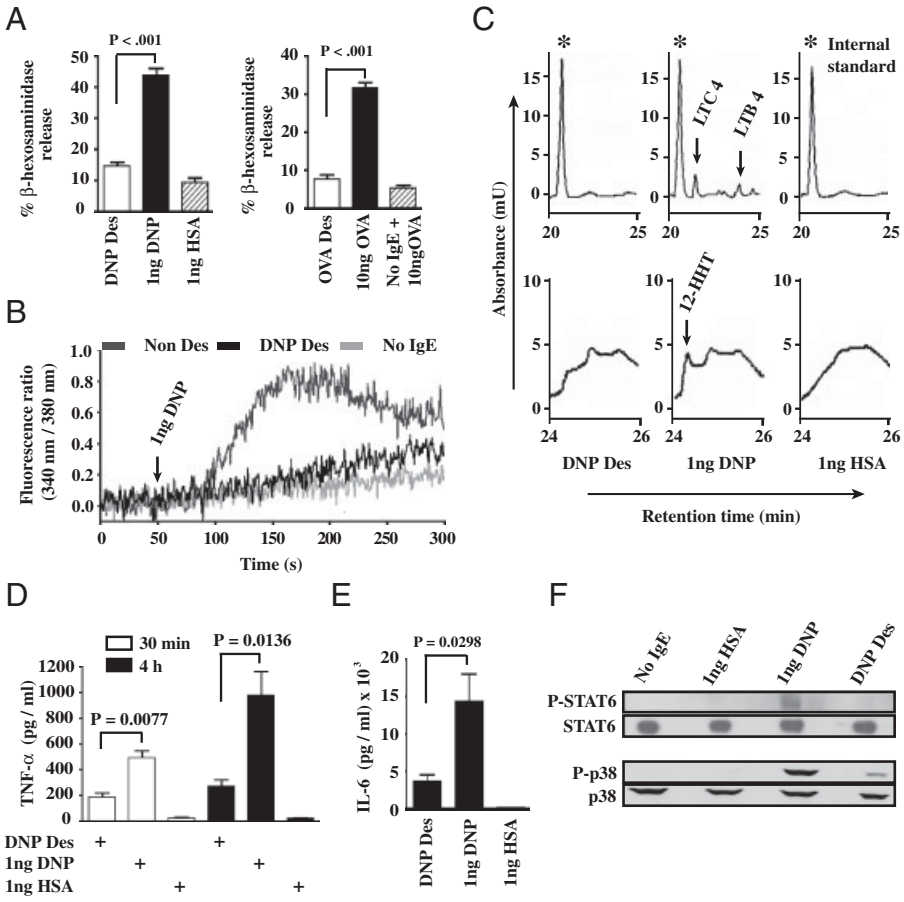

Figure 2. Rapid desensitization impairs early- and late-phase activation responses in BMMCs. (A) Percentage of β-hexosaminidase release after desensitization (DNPDes or OVADes) or DNP-HSA or OVA challenge (1 ng DNP or 10 ng OVA). Negative controls were 1ng HSA or No IgE+10 ng OVA. (B) Calcium flux when 1 ng DNP-HSA is added to cells treated as indicated. (C) RP-HPLC analysis of arachidonic acid products in supernatants of cells treated as indicated, with retention times for PGB_2 (internal standard), LTC_4, LTB_4 and 12-HHT of 20.6, 21.4, 23.7 and 24.4 min, respectively. (D) TNF-α secretion (E) IL-6 secretion and (F) phospho-STAT6 and phospho-p38 MAP kinase Western blots of cells treated as indicated. (A, D and E) data are expressed as mean+SEM of three independent experiments, $p<0.05$ was considered to be significant. (B, C and F) Data are representative of three independent experiments. DNPDes: rapid desensitization to DNP; OVADes: rapid desensitization to OVA.

Because calcium mobilization is key to arachidonic acid metabolization and generation of prostaglandins and leukotrienes, we studied arachidonic acid products. Thirty minutes after 1 ng DNP-HSA challenge, cell supernatant was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC); cysteinyl leukotriene C4 (LTC_4), leucotriene B4 (LTB_4), and 12(*S*)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid (12-HHT) were identified with retention times of 21.4, 23.7 and 24.4 min, respectively, with prostaglandin B2 (PGB_2) as an internal standard. In contrast, LTB_4, LTC_4 and 12-HHT were not detected in rapidly desensitized cell supernatants or in cells treated with 1 ng HSA (see Fig. 2C). This result indicates a lack of arachidonic acid metabolization with desensitization.

Other proinflammatory mediators are released from mast cells upon activation, such as TNF-α and IL-6 cytokines. Pre-formed TNF-α is released upon IgE stimulation in the early-phase response, while secretion of de novo synthesized TNF-α and IL-6 production occurs several hours post-stimulation, in the late-phase response. Because early-phase activation events may influence late-phase responses, and because desensitization may affect early and late-phase responses differently, we studied TNF-α, a product of mast cell responses in both phases, and IL-6, a cytokine not typically stored but produced in the late phase. Pre-formed TNF-α released with 1 ng DNP-HSA challenge was 490 pg ± 15%, while in rapid-desensitized cells the release was 185 pg ± 23%, a significant 62% reduction (see Fig. 2D, white bars). During the late-phase response, 4 h after activation or desensitization, the release of newly generated TNF-α from DNP-activated cells was 978 pg ± 23%, while rapid-desensitized cells released 272 pg ± 22%, a significant 72% reduction (see Fig. 2D, black bars). The production of IL-6 assessed 4 h after activation or desensitization (see Fig. 2E) was 14362 pg ± 42% and 3665 pg ± 35%, respectively, showing a 75% reduction. Those results indicate that desensitization impaired early- and late-phase mast cell responses.

It has been reported that STAT6 plays a pivotal role in antigen/IgE/FcεRI-mediated cytokine release from mast cells and that STAT6 phosphorylation occurs not only through the JAK-STAT

pathway after IL-4 receptor activation but also after antigen crosslinking of FcεRI/IgE [18]. Since our previous studies showed that STAT6-null BMMCs from BALB/c and C57BL/6 mice could not be desensitized [16], we explored how rapid desensitization targeted STAT6. We evaluated STAT6 phosphorylation in DNP-HSA-activated and desensitized cells and observed that STAT6 was not phosphorylated with rapid desensitization (see Fig. 2F).

Since FcεRI-mediated mitogen-activated protein kinases (MAPKs) activation leads to gene transcription of several cytokines [19, 20], we next examined the levels of phosphorylation of p38 MAPK in DNP-HSA-activated and desensitized cells (see Fig. 2F). As expected by the low levels of TNF-α and IL-6 production, p38 MAPK phosphorylation was inhibited by rapid desensitization, indicating that molecular events leading to cytokine gene transcription were inhibited during rapid desensitization.

Duration of, and antigen requirements for, hypo-responsiveness after rapid desensitization

Because the duration of desensitization may depend on the presence of bound and soluble antigen, we determined the duration of, and antigen requirements for, maintaining hypo-responsiveness after desensitization.

Cells challenged with 1 ng DNP-HSA at 10 min, 2 h and 4 h after desensitization, remained hypo-responsive with a 20% β-hexosaminidase release (see Fig. 3A, first bar of each time group of bars). Treatment of desensitized cells with ionomycin at 10 min, 2 h or 4 h after desensitization, resulted in high levels of β-hexosaminidase release (see Fig. 3A, second bar of each time group of bars), indicating that desensitized cells were not mediator-depleted. Further time points were not pursued due to diminishing cell viability after 6 h (from 91 to 83% viability 4 h after desensitization (100 min)). This decrease in cell viability was attributed to low volume (10^6 cells in 50–100 μL) and IL-3 and CO_2 depletion.

We then considered the possibility that desensitized BMMCs could remain hypo-responsive to further stimulation due to the excess of soluble antigen. Washed and non-washed desensitized cells responded similarly to challenge (see Fig. 3B), indicating that once hypo-responsiveness was achieved the presence of soluble antigen was not required for maintaining desensitization.

Rapid desensitization inhibits FcεRI internalization but does not impair specific activation

Internalization of antigen/IgE/FcεRI complexes has been demonstrated after cell activation [21, 22], and it has been suggested that mast cell hypo-responsiveness to low antigen doses is due to internalization of antigen-bound receptors [12]. We wanted to determine the fate of the antigen/IgE/FcεRI complex with desensitization.

We analyzed surface expression of FcεRIα and IgE in rapid-desensitized cells, in cells challenged with 1 ng DNP-HSA or with 1 ng HSA, and in non-sensitized cells. Surface expression levels of FcεRIα and IgE in desensitized cells were similar to those of cells challenged with 1 ng HSA and significantly higher than in activated cells (see Fig. 4A), indicating the impairment of internalization of IgE and FcεRIα.

Since most of the IgE/FcεRI complexes remained on the cell surface, we sought to determine whether anti-IgE could crosslink free IgE on desensitized cells. DNP-desensitized cells released β-hexosaminidase when treated with anti-IgE (see Fig. 4B), indicating that unbound IgE was available for crosslinking and remained accessible.

To assess the fate of the desensitizing antigen, we used Alexa Fluor 488-conjugated OVA and followed its localization in activated and desensitized cells (see Fig. 4C). A cross-sectional view of the intracellular compartment revealed that cells challenged with 50 ng of fluorescently labeled OVA showed large internalized aggregates, as confirmed by other researchers [23]. In contrast, OVA-desensitized cells showed fewer and smaller fluorescent aggregates, and their visual appearance was similar to that of cells challenged at 4°C, in which crosslinked receptors were not internalized and appeared with small aggregates bound to the membrane.

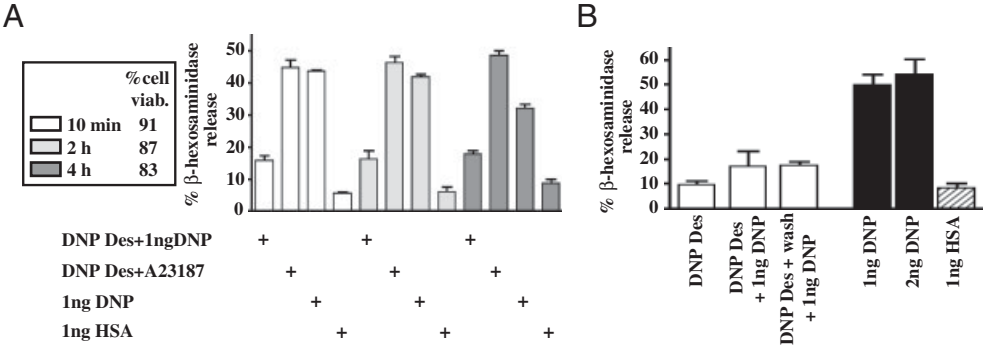

Figure 3. Duration of, and antigen requirements for, hypo-responsiveness after rapid desensitization. (A) Percentage of β-hexosaminidase release from cells sensitized overnight with anti-DNP IgE in response to the indicated treatments at various time points after desensitization. (B) Maintenance of desensitization with or without washing before challenge with 1 ng DNP-HSA. Data are expressed as mean+SEM of three independent experiments.

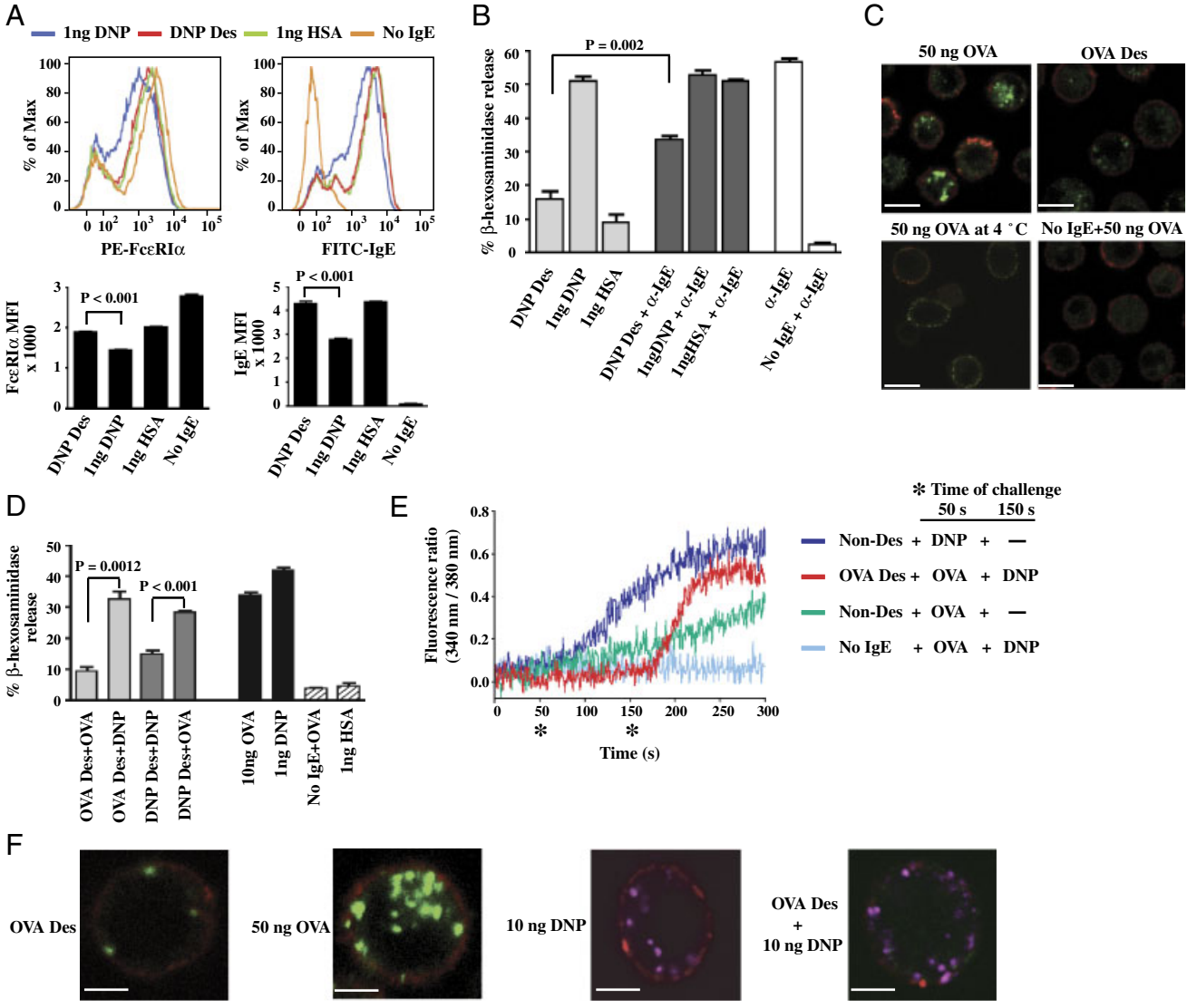

Figure 4. Antigen/IgE/FcεRI complex internalization is inhibited during BMMC rapid desensitization but does not impair specific activation. (A) Cells sensitized overnight with anti-DNP IgE or non-sensitized cells used as a control (No IgE) were treated as indicated. Representative histograms with FcεRIα and IgE surface expression (upper panel) and mean fluorescence intensities are shown (lower panel). (B) Percentage of β-hexosaminidase release in response to the indicated treatments. (C) Anti-OVA IgE sensitized cells or non-sensitized cells (No IgE) were treated as indicated and visualized by confocal microscopy. Cholera Toxin subunit B-Alexa Fluor 555 (red), OVA-Alexa Fluor 488 (green). Fields were obtained from one experiment and are representative of four independent experiments. Scale bar = 8.5 μm, original magnification 630 ×. In (D, E and F) cells were sensitized with both anti-DNP IgE and anti-OVA IgE and non-sensitized cells were used as negative control for OVA activation (No IgE). Percentage of β-hexosaminidase release (D) and calcium flux (E) of cells in response to the indicated treatments. Data in (A, lower panel, B and D) are expressed as mean+SEM of three independent experiments. Statistical significance was determined using Student's unpaired two-tailed *t* test, $p<0.05$ was considered to be significant. (F) Confocal microscopy of cells treated as indicated. Cholera Toxin subunit B-Alexa Fluor 555 (red), OVA-Alexa Fluor 488 (green) and DNP-DyLight 649 (purple). Fields were obtained from one experiment and are representative of three independent experiments. Scale bar = 3 μm, original magnification 630 ×.

Since desensitized cells were hypo-responsive to further triggering doses of the same antigen, we studied the response to a second triggering antigen. Cells sensitized with anti-DNP IgE and anti-OVA IgE were desensitized to OVA or to DNP and then challenged with triggering doses of DNP-HSA or OVA, respectively. Cells desensitized to OVA responded (β-hexosaminidase release) to a triggering dose of 1 ng DNP-HSA, and cells desensitized to DNP responded to a triggering dose of 10 ng OVA (see Fig. 4D), indicating that mediators were not depleted after desensitization to one antigen and that desensitization disabled the specific response only to the desensitizing antigen.

We then analyzed the specificity of the calcium responses. Cells desensitized to OVA had impaired calcium influx when triggered with 10 ng OVA, but the influx was restored by a triggering dose of 1 ng DNP-HSA (see Fig. 4E, red line), indicating that the calcium response was compartmentalized by specific antigen.

We then analyzed specificity using confocal microscopy (see Fig. 4F). OVA-desensitized cells showed low internalization of labeled OVA antigen (green) as compared to the larger aggregates seen in OVA-activated cells. When OVA-desensitized cells were challenged with DNP-HSA (purple), the amount of internalization was comparable to that of DNP-HSA activated cells, indicating that desensitization left unaffected the specific mechanisms of cell activation and receptor internalization.

Discussion

Our understanding of IgE desensitizations has been limited by the paucity of in vitro mast cell models providing quantitative and qualitative insight into the early and late cell responses. Here, we present an in vitro 11-step model of mouse BMMC rapid IgE desensitization under physiologic calcium conditions and characterize its kinetics, effectiveness, antigen specificity and receptor internalization-associated events.

We showed that desensitization is a dynamic process in which each step provides a platform for the next level of response reduction and that once desensitized, mast cells remain hyporesponsive to further antigen challenges. Increasing the target antigen dose for desensitization decreased the hypo-responsiveness, suggesting that additional steps and/or time between steps might help to achieve better desensitization, thus underscoring the critical relationship between IgE sensitization and the target antigen dose for desensitization. In human desensitizations the level of IgE sensitization varies and is unknown for each patient and the target dose used for desensitization is empirical, which impacts its safety [4, 5, 8].

The mechanism of desensitization is not fully understood and we have observed that low antigen doses induce small amounts of extracellular calcium flux, indicating the mobilization of endoplasmic reticulum stores, enabling functional CRAC channels to open [17]. The sequential delivery of low antigen doses during desensitization may provide continued low levels of calcium entry with conformational changes of CRAC and other calcium-related channels locking further calcium entry and blocking signal transduction. Because calcium entry is clearly specifically impaired in our model, since a second non-desensitizing antigen allowed restoration of calcium flux, membrane compartmentalization may be required to exclude signal transduction molecules around desensitized receptors.

We observed that in desensitized cells, phosphorylation of STAT6 and p38 MAP kinase was impaired and consequently TNF-α and IL-6 production was diminished. Since earlier studies indicated that STAT6-null BMMCs could not be desensitized [16], it is possible that STAT6 activity is required for desensitization, via a pathway different from the one leading to the acute and late activating responses. Our system is limited by the fact that BMMCs are cultured in IL-3, which may affect cytokine production [24]. Nonetheless, this may have an important correlate in human desensitizations since our group has not observed delayed reactions in desensitized patients, confirming that the inhibition of mast cell activation during desensitization prevented later hypersensitivity reactions [4, 5].

Maintenance of hypo-responsiveness in desensitized cells was not sustained by the presence of an excess of soluble antigen since washed cells remained desensitized. It is possible that bound antigen is equilibrated in desensitized cells. Earlier studies [12, 13] suggested that the hypo-responsiveness induced by desensitization was due to internalization of antigen/IgE/FcεRI complexes and that the lack of available IgE renders the cells refractory to further stimulation. In contrast, we show here that, unlike activation, internalization of IgE and FcεRI is impaired during specific desensitization (Fig. 4A) and that desensitized cells can be triggered by anti-IgE, since unbound IgE remains accessible and is available for crosslinking (Fig. 4B). Saturating doses of IgE in a co-culture system and the use of higher antigen doses [12] may promote internalization while low doses may redistribute antigen-bound receptor at the membrane level. Moreover, others have shown that low doses of antigen induce antigen-crosslinked receptors to remain mobile on the cell surface [25]. In addition, microscopy studies gave us the opportunity to directly look into antigen localization after desensitization.

Previous studies in calcium-depleted conditions have shown that cells desensitized to one antigen could not be triggered with a non-desensitizing second antigen [26], possibly through a disabling mechanism involving syk. Due to the amount of IgE sensitization and low antigen doses used in our model, we could not detect syk phosphorylation. Our findings indicate that the mast cell-activating machinery was intact for a non-desensitizing antigen action, since no mediator depletion occurred with desensitization, calcium flux was restored in desensitized cells when challenged with a non-desensitizing antigen and microscopic analysis confirmed that rapid desensitization is antigen specific and does not induce anergy [27].

While we do not know the exact mechanism that could explain this inhibition of receptor internalization during desensitization, it is possible that the mobility of antigen/IgE/FcεRI complexes and membrane re-arrangement could prevent their internalization, as shown by others with low doses of multivalent antigen [25]. In addition, receptors engaged with low doses of antigen could be segregated into different compartments, preventing access to phosphorylating molecules. Inhibitory phosphatases such as SHP-1 may not be excluded from those compartments, thus preventing phosphorylation of key molecules required for signal transduction. A time course study of SHP-1 phosphorylation in RBL-2H3 cells [28] has shown a peak at 1 min of FcεRI crosslinking and a gradually decline within 10 min. Our initial results indicated a lack of phosphorylation at 100 min. (data not shown). Further studies are planned to look for phosphorylation of SHP-1 and other ITIM-bearing molecules [29, 30] at each step of the desensitization protocol since it may be transient.

In conclusion, this model of rapid IgE desensitization is effective and reproducible and provides an optimal dose–time relationship, leading to almost complete abrogation of early- and late-phase activation events. This model of antigen-specific desensitization disables the specific response to one antigen

but keeps the cell machinery unaffected, unlike non-specific desensitization. Most importantly, we show here that specific rapid desensitization inhibits internalization of the antigen/IgE/FcεRI complexes.

The lack of severe anaphylactic reactions in our previous clinical reports [4, 5], including hundreds of desensitizations using a modified protocol, illustrates a profound inhibition of acute and delayed mast cell activation. These studies provide proof of concept for the effectiveness and specificity of human desensitizations.

Materials and methods

Cell culture

BMMCs derived from femurs of male BALB/c mice 8–12 wk old (Jackson Laboratory) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1% *Penicillin-Streptomycin*, 0.1 mM MEM nonessential amino acids (all from Sigma-Aldrich) and 10 ng/mL of IL-3. IL-3 was obtained from supernatants of 293T cells expressing mouse IL-3 [31, 32].

Activation and rapid desensitization of BMMCs to DNP and OVA antigens

DNP antigen: Cells were sensitized overnight with anti-DNP IgE (0.25 μg/10^6 cells/mL). The next day, cells were washed to eliminate possible excess of unbound IgE, resuspended in 50 μL of fresh medium without IL-3 and placed at 37°C. For desensitization, cells were treated as per Table 1 (rapid desensitization protocol), and 10 min after the last DNP-HSA addition, placed on ice for β-hexosaminidase release assay. For activation, cells were challenged with 50 μL of DNP-HSA at 20 pg/μL (1 ng DNP) and for control, with 50 μL of HSA at 20 pg/μL (1 ng HSA), and after 10 min, placed on ice for β-hexosaminidase release assay. β-Hexosaminidase release assay was performed as previously described [16].

OVA antigen: Same described method used for DNP antigen, but with overnight sensitization performed with murine post-immunization serum with OVA-specific IgE (0.25 μg/10^6 cells/mL) (anti-OVA IgE). For activation, 50 μL of OVA at 200 pg/μL (10 ng OVA) was used. For control, 50 μL of OVA at 200 pg/μL was added to cells without anti-OVA IgE overnight incubation.

For specificity experiments, cells were sensitized overnight with 0.25 μg/10^6 cells/mL of both anti-DNP IgE and anti-OVA IgE.

Challenge with anti-IgE

After cells were desensitized or challenged with DNP or HSA, we treated them with 100 ng of rat anti-mouse IgE (clone R35-72 from BD Pharmingen). For control, cells incubated overnight with or without anti-DNP IgE were also treated with 100 ng of rat anti-mouse IgE.

Measurement of intracellular calcium

Desensitized, non-desensitized and non-IgE treated cells were washed and resuspended in HBSS containing 1 mM CaCl2, 1 mM MgCl2 and 0.1% BSA (Buffer A). Cells were then loaded with 2.5 μM Fura-2AM (Molecular Probes) in the presence of 2.5 mM probenecid for 30 min at 37°C. After being labeled, cells were washed and resuspended in cold Buffer A (0.5×10^6/mL). Fluorescence output was measured with excitation at 340 and 380 nm in the F-4500 Fluorescence Spectrophotometer (Hitachi), and the relative ratio (R) of fluorescence emitted at 510 nm was recorded. For all fluorescence ratios to start at zero, the first fluorescence value of each sample was subtracted from all its subsequent fluorescence values.

RP-HPLC

After desensitization or challenge, cell supernatants were collected and LTB_4, LTC_4 and 12-HHT were measured by RP-HPLC following a published protocol [33]. Briefly, samples were applied to a C18 Ultrasphere RP column (Beckman Instruments) equilibrated with a solvent consisting of methanol/ACN/water/acetic acid (10:15:100:0.2, v/v), pH 6.0 (Solvent A). After injection of the sample, the column was eluted at a flow rate of 1 mL/min with a programmed concave gradient to 55% of the equilibrated Solvent A and 45% of Solvent B (100% methanol) over 2.5 min. After 5 min, Solvent B was increased linearly to 75% over 15 min and maintained at this level for an additional 15 min. The UV absorbance at 280 and 235 nm and the UV spectra were recorded simultaneously. PGB_2 was used as an internal standard.

TNF-α and IL-6 measurement

After desensitization or challenge, TNF-α and IL-6 contents in cell-free supernatants were estimated using a mouse TNF-α or IL-6 ELISA kits (eBioscience), either 30 min or 4 h after activation or desensitization, according to the manufacturer's protocol.

Duration of rapid desensitization

Cells were rapid desensitized as per Table 1. After desensitization (nearly 2 h) cells were maintained for 10 min, 2 hours, or 4 hours at 37°C. After each time period, 1 ng of DNP-HSA or 25 μL of calcium ionophore A23187 (Sigma-Aldrich) 10 μM was added. Non-desensitized cells were kept at 37°C and challenged with 1 ng of DNP-HSA or 1 ng HSA at the same time points as for

desensitized cells. The total time for all cells at 37°C, since rapid desensitization protocol lasts nearly 2 h, was 6 h. Cell viability was assessed by trypan blue dye exclusion.

Immunoblot analysis

After desensitization or challenge, cells were collected and washed with cold PBS. Pellets were lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktails (Roche). Total protein lysates were subjected to SDS-PAGE on a 4–12% polyacrylamide gel and transferred to a nitrocellulose membrane (both from Invitrogen). Membranes were blotted with anti-Phospho-STAT6 (phosphotyrosine 641) and anti-STAT6 from Sigma-Aldrich or with anti-Phospho-p38MAP kinase and anti-p38αMAP kinase from Cell Signaling. Signal detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Flow cytometry analysis

After desensitization or challenge, cells were placed at 4°C, then washed and resuspended in PBS containing 0.5% BSA and 0.05% sodium azide at 4°C and incubated with anti-FcγRI/II mAb (eBioscience) for 20 min on ice to block Fcγ receptors. Cells were then incubated with 5 μg/mL FITC rat anti-mouse IgE (BD Biosciences) or 2 μg/mL PE Armenian hamster anti-mouse FcεRIα (eBioscience) or with the recommended isotype controls. Cells were analyzed on a BD Biosciences FACSCanto flow cytometer, using FACSDiva acquisition software and FlowJo analysis software.

Confocal microscopy

Antigens used were Alexa Fluor 488-conjugated OVA (Molecular Probes) and DyLight Fluor 649-conjugated DNP, labeled with DyLight 649 NHS Ester (Thermo Scientific). Due to detection limitations, OVA activation dose was 50 ng, DNP activation dose was 5 ng and the rapid OVA desensitization protocol was consequently adjusted based on the volumes used in the protocol in Table 1 but at higher concentrations. After desensitization or challenge, cells were washed and resuspended in cold PBS. Cells were transferred onto poly-L-lysine-coated round cover slips for 20 min at 4°C and then fixed with 4% paraformaldehyde in PBS for 10 min at 4°C. After three washes with PBS, cells were incubated with cholera toxin subunit B-Alexa Fluor 555 conjugate (Molecular Probes) 1:500 in PBS for 10 min at 4°C, washed three times with PBS and mounted using an aqueous mounting medium (15% wt/v polyvinyl alcohol, 33% v/v glycerol, 0.1% azide). Images were collected sequentially using a 63× plan Apo NA 1.4 objective on Leica SP5X laser scanning confocal system attached to an inverted Leica DMI6000 microscope.

Statistical analyses

Data are expressed as mean ± SEM using Prism4. Statistical significance was determined using Student unpaired two-tailed *t* test. $p<0.05$ was considered to be significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **12-HHT:** 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid · **BMMCs:** BM-derived mast cells · **CRAC:** calcium-release-activated calcium · **FcεRI:** high-affinity receptor for IgE · **HSA:** human serum albumin · **LTB_4:** leucotriene B4 · **LTC_4:** cysteinyl leukotriene C4 · **PGB_2:** prostaglandin B2 · **syk:** spleen tyrosine kinase

Full correspondence: Dr. Mariana Castells, Brigham and Women's Hospital- Smith Building, Room 626D 1 Jimmy Fund Way Boston, MA 02115, USA
Fax: +1-617-525-1310
e-mail: mcastells@partners.org

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REVIEW

Desensitization regimens for drug allergy: state of the art in the 21st century

A. Liu*, L. Fanning*, H. Chong*, J. Fernandez*, D. Sloane, M. Sancho-Serra and M. Castells
Drug Desensitization Unit, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

Clinical & Experimental Allergy

Correspondence:
M. Castells, MD PhD, Drug Desensitization Unit, Harvard Medical School, Brigham and Women's Hospital, Boston, MA 02115, USA.
E-mail: mcastells@partners.org

Summary
Adverse reactions to drugs are increasingly being recognized as important contributions to disease in their own right as well as impediments to the best treatment of various conditions, including infectious, autoimmune, and neoplastic maladies. Rapid drug desensitization (RDD) is an effective mechanism for safely administering important medications while minimizing or entirely circumventing such adverse reactions in sensitized patients. We reviewed the literature on RDD in the last 10 years, including our experience from the Brigham and Women's Hospital Desensitization Program with hundreds of patients desensitized to a broad variety of drugs. RDD in our programme has been uniformly successful in patients with hypersensitivity reactions to antibiotics, chemotherapeutics, and monoclonal antibodies. Any reactions that occur during desensitization are generally much less severe than the initial hypersensitivity reaction to the drug, and patients have received the full dose of the desired medication 99.9% of the time out of (796) desensitizations. To date, there have been no fatalities. RDD is a safe and highly effective method for treating sensitized patients with the optimal pharmacologic agents. Its use should be expanded, but because patient safety is paramount, protocols must be created, reviewed, and overseen by allergist–immunologists with special training and experience in modern techniques of desensitization.

Introduction

Innovation to address the need to treat patients with a wide variety of common and important diseases, including infections, malignancies, and arthritides, has brought forth a number of novel pharmacologic agents. With a larger gamut of drugs, clinicians must decide which agent is the best for a particular patient with a given disease, personalizing treatment. Adverse drug reactions, however, are frequently encountered and threaten to relegate the patient to a secondary therapy. Some of these reactions are mast cell-mediated hypersensitivity reactions (HSRs), a subset of which occur through an IgE-dependent mechanism, and are thus true allergies. Rapid drug desensitization (RDD) is a technique that uses protocols that induce temporary tolerance to a drug, allowing a patient with such a drug hypersensitivity to receive the optimal agent for his or her disease.

General principles and proposed mechanisms of rapid drug desensitization

The cell targets for rapid desensitization are thought to be mast cells and possibly basophils. Once mast cells and basophils are sensitized with specific IgE to medications, exposure to the allergen medication can cause the sudden systemic release of inflammatory mediators from activated mast cells, leading to anaphylaxis. RDD is a process by which mast cells are rendered hypo-responsive to a medication allergen by providing temporary tolerization for drug hypersensitive patients, protecting them from anaphylaxis. Desensitization protocols typically include the incremental, stepwise-fashion administration of increasing amounts of the medication allergen without eliciting life-threatening symptoms [1–3].

Research into the mechanisms of RDD has focused largely on patients with a positive skin test to the culprit medication, indicating that mast cells (likely through drug-specific IgE) are the main cells responsible for these reactions. After rapid desensitization, specific skin test reactivity is abolished, indicating that the allergen is no longer able to trigger skin mast cell activation and that systemically distributed mast cells have lost the ability to release mediators [4]. Mast cells activated by antigen cross-linking of IgE-bound FcεRI receptors display aggregation of these receptors, recruitment and activation of target molecules, calcium mobilization, degranulation, arachidonic acid metabolism, and cytokine and chemokine gene transcription [5, 6]. Three non-mutually

*Contributed equally to this review.

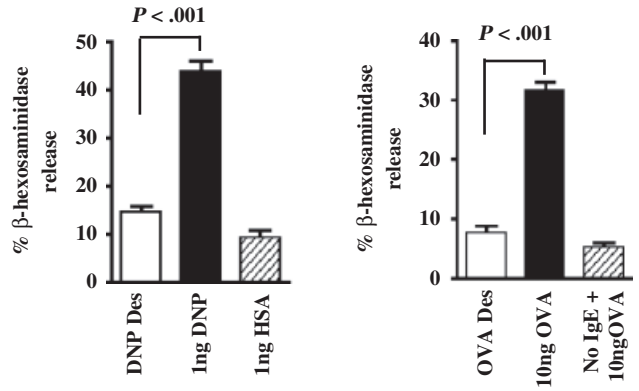

Fig. 1. Desensitization inhibits the release of mast cell granule mediators. Release of granule mediators (β-hexosaminidase) after DNP and ovalbumin (OVA) antigens activations in bone marrow mouse mast cells and after desensitization (DNP desensitization and OVA desensitization). Adapted from Sancho-Serra et al. [67].

exclusive hypotheses explaining how RDD could impair mast cell activation have been articulated: (1) depletion of activating signal transduction components such as syk kinase, (2) sub-threshold depletion of mediators, and (3) internalization of FcεRI through progressive cross-linking at a low antigen concentration [7, 8].

Ubiquitination of syk after prolonged exposure to sub-threshold doses of antigen is one mechanism for inducing unresponsiveness of basophils and mast cells, but this process is unlikely to explain the efficacy of rapid desensitizations because the antigen exposure during desensitization does not allow sufficient time for this to occur.

An *in vitro* model of antigen-specific, rapid mast cell/IgE desensitization in the presence of physiologic levels of calcium was developed (Fig. 1). Increasing doses of antigen delivered at fixed time intervals induced a highly specific and prolonged hypo-responsiveness to triggering doses of the desensitizing antigen. The release of granules mediators such as β-hexosaminidase and the metabolism of arachidonic acid products such as prostaglandins and leukotrienes were inhibited by desensitization (Figs 1 and 2). The desensitization process was achieved by incremental doses administered at fixed time intervals and did not produce a slow release of mediators as mast cells responded well to further allergen stimulation after each of the desensitizing doses (Fig. 3). As long as the antigen was maintained, the desensitization was maintained, indicating that the presence of allergen was necessary (Fig. 4). Mast cells desensitized to DNP antigen demonstrated almost complete inhibition of release of pre-formed and newly generated TNF and IL-6, explaining why patients are not at a risk for a delayed reaction after rapid desensitization, as late-phase mediator generation does not occur (Fig. 5). When mast cells were sensitized to both DNP and OVA antigens, OVA-desensitized cells responded fully to ONP, proving antigen specificity and providing evidence that the activating signal transduction pathways

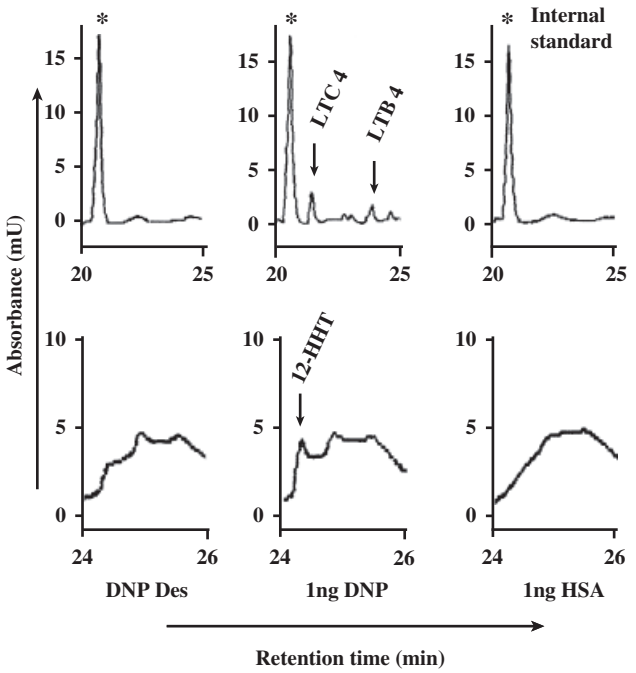

Fig. 2. Desensitization inhibits the metabolism of arachidonic acid and the generation of prostaglandins and leukotrienes. Upper panel: LTC4 and LTB4 peaks during activation and desensitization. Lower panel: 12 HHT (prostaglandin pre-cursor) during activation and desensitization. Adapted from Sancho-Serra et al. [67].

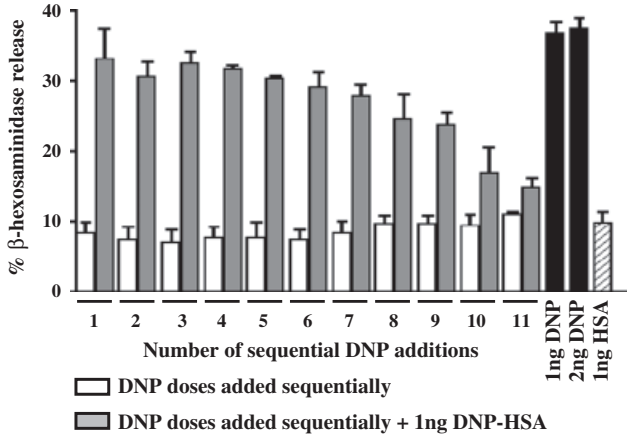

Fig. 3. Desensitization of BMMC required multiple sequential doses and does not induce mediator depletion. Release of granule mediators (β-hexosaminidase) after sequential doses of DNP antigen (1 through 11) ± 1 activating dose of DNP. Adapted from Sancho-Serra et al. [67].

are intact for a second allergen (Fig. 6). Therefore, the hypothesis that activating signalling molecules are exhausted during rapid desensitization is not supported. Importantly, antigen-specific IgE bound to the α-chain of FcεRI remained at the cell surface after rapid desensitization, indicating that the lack of reactivity during desensitization was not due to the disappearance of surface IgE and FceRI when bound to small doses of antigen (Fig. 6). Thus, although these models recapitulated the profound

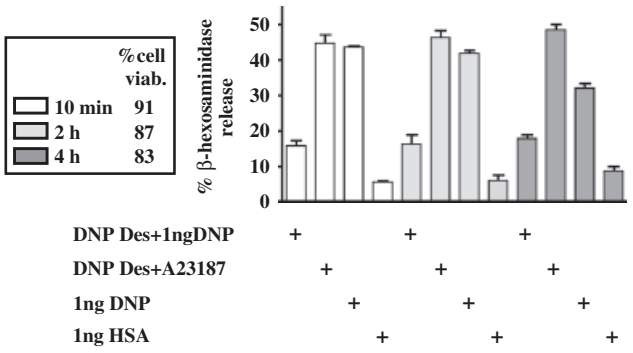

Fig. 4. Desensitization is maintained as long as antigen is present. Release of granule mediators (β-hexosaminidase) after desensitization, 2 and 4 h later. Adapted from Sancho-Serra et al. [67].

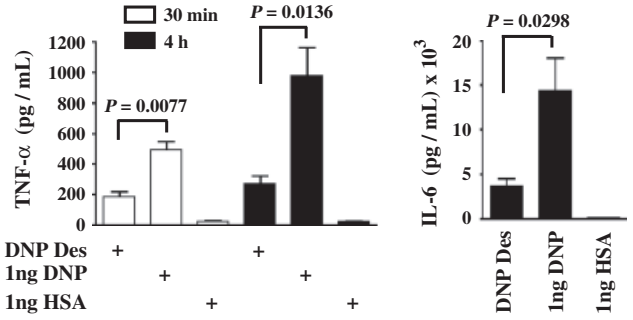

Fig. 5. Desensitization inhibits acute- and late-phase cytokines TNF- and IL-6. TNF-α and IL-6 were measured in the supernatants after the activation and desensitization of BMMC. Adapted from Sancho-Serra et al. [67].

inhibition of acute and delayed mast cell responses that provide protection against anaphylactic reactions, and provided the basis for an adapted protocol that has been applied to hundreds of successful desensitizations, they have not shed light on the mechanisms actually responsible for mast cell hypo-responsiveness [2, 3].

General principles of rapid drug desensitization

The BWH Desensitization Program devised a 12- to 20-step standard protocol based on an *in vitro* mouse mast cell model, in which unresponsiveness to a triggering antigen dose was achieved by delivering doubling doses of antigen at fixed time intervals starting at 1/1000 the final dose [9]. The most commonly used protocol has 12 steps, using three solutions at escalating rates (Fig. 7). Patients who have had severe anaphylactic reactions to the agent of choice or who have reacted early in the standard 12-step desensitization may experience fewer symptoms if desensitized using a 16-step protocol, which adds another bag containing 1/1000th of the full dose. The use of a 16-step (four bags) or a 20-step (five bags) protocol is reserved for high-risk patients. Common side-effects include flushing, warmth, pruritus, erythema, and urticaria, and patients are cautioned about the low but real risk of anaphylaxis.

Our standardized desensitization protocol has been described previously [1]. Routine premedication consists of a single dose each of diphenhydramine and famotidine. Specific tailoring of a protocol may include the addition of aspirin, montelukast, or glucocorticoids to the pretreatment regimen based on previous symptoms of flushing or throat itching. β-adrenergic blocking medications are held for 24 h before desensitization. An essential point is that a thoughtful approach to drug hypersensitivity and RDD requires more than the standard desensitization protocol. No rigid algorithm, no matter how widely applicable, will suffice. Instead, our dynamic and flexible practice is to follow these steps:

1. evaluate the patient, attempting to characterize the nature of a patient's adverse reaction,
2. determine the likelihood that RDD will be effective and safe,
3. apply or design a reasonable RDD protocol (often using our standard 12-step protocol as a starting place),
4. collect information about how the patient responds to each desensitization and modify the protocol as needed:

(a) adding, subtracting, or changing premedications,
(b) changing the number of steps in the protocol,
(c) altering the rate or time of one or more steps, and
(d) some combination of these.

Thus, drug desensitization truly begins with an analysis of the patient's HSR, design and testing of an initial desensitization protocol, and adjustment of this protocol in an iterative fashion based on the patient's response. Adverse drug reactions inducing a type I hypersensitivity reaction, whether IgE or non-IgE mediated, are eligible for rapid desensitizations. The symptoms of these reactions include cutaneous (flushing, pruritus, urticaria or angioedema, maculopapular rash), respiratory (nasal congestion, sneezing, wheezing, shortness of breath, cough, O_2 desaturation), gastrointestinal (nausea, vomiting, diarrhoea, abdominal pain, bloating), cardiovascular (chest pain, tachycardia, presyncope syncope, hypertension, hypotension, EKG changes), neuromuscular (sense of impending doom, disorientation/hallucination, visual disturbances, unusual taste, back pain, numbness/weakness), and/or throat tightness during the infusion or shortly after the administration of these medications. Because rapid desensitization does not result in long-term tolerance, patients need to be re-desensitized each time they are exposed to the allergenic medication. If the medication is maintained at pharmacological levels by daily

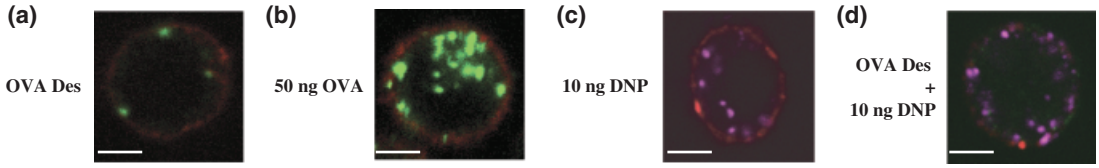

Fig. 6. Desensitization is antigen specific and prevents the internalization of the antigen/IgE/FceRI complex. Internalization is seen after ovalbumin (OVA) activation (b), but not with desensitization (a), and DNP is internalized after activation in OVA-desensitized cells, which is comparable to that of DNP-HSA active cells (d). Adapted from Sancho-Serra et al. [67].

Name of medication:		***Remicade***					
Target dose (mg)				800			
Standard volume per bag (mL)				250			
Final rate of infusion (mL/hr)				80			
Calculated target concentration (mg/mL)				3.2			
Standard time of infusion (min)				187.5			
					Total mg per bag	Amount of bag infused (mL)	
Solution 1	250	mL of	0.032	mg/mL	8.000	9.25	
Solution 2	250	mL of	0.320	mg/mL	80.000	18.75	
Solution 3	250	mL of	3.175	mg/mL	793.704	250.00	
*** PLEASE NOTE ***		The total volume and dose dispensed are more than the final dose given to patient					
			because the initial solutions are *not completely infused*				
Step	**Solution**	**Rate (mL/h)**	**Time (min)**	**Volume infused per step (mL)**	**Dose administered with this step (mg)**	**Cumulative dose (mg)**	Fold increase per step
1	1	2.0	15	0.50	0.0160	0.0160	–
2	1	5.0	15	1.25	0.0400	0.0560	2.5
3	1	10.0	15	2.50	0.0800	0.1360	2
4	1	20.0	15	5.00	0.1600	0.2960	2
5	2	5.0	15	1.25	0.4000	0.6960	2.5
6	2	10.0	15	2.50	0.8000	1.4960	2
7	2	20.0	15	5.00	1.6000	3.0960	2
8	2	40.0	15	10.00	3.2000	6.2960	2
9	3	10.0	15	2.50	7.9370	14.2330	2.480325
10	3	20.0	15	5.00	15.8741	30.1071	2
11	3	40.0	15	10.00	31.7482	61.8553	2
12	3	80.0	174.375	232.50	738.1447	800.0000	2
	Total time (min) =		339.375	= 5.66 h			

Fig. 7. A standard 12-step, three-bag desensitization protocol from the Brigham and Women's Hospital Rapid Drug Desensitization Program.

administration, such as with aspirin desensitization intended for daily use for cardio-protection or during an antibiotic course in which the antibiotic is given at regular intervals, the desensitized state is maintained. *In vitro* experiments confirm that maintaining the presence of the allergen preserves mast cell unresponsiveness. For medications given at intervals significantly greater than their half-lives, such as monoclonal antibodies and chemotherapeutic agents, desensitization needs to be repeated for each administration.

Below, we summarize experience with rapid desensitization to four different classes of drugs: antibiotics, taxane chemotherapy agents, platin-based chemotherapeutic agents, and monoclonal antibodies and other miscellaneous medications.

Rapid drug desensitization to antibiotics

Despite a wide selection of antibiotics available for the treatment of inpatient and outpatient infections, a single

antibiotic often emerges as the preferred choice in a given situation. Not infrequently, the antibiotic chosen is one to which the patient has a history of HSR. Drug resistance, prohibitive intolerances, limited bactericidal or bacteriostatic activity, and poor bioavailability of alternatives pose a risk of uncontrolled infection that outweighs those of desensitization. Unlike chemotherapy and monoclonal antibodies, antibiotics are usually administered over a course of several days to weeks in doses scheduled 6–24 h apart. When considering antibiotic desensitization, one must verify that regular dosing of the antibiotic for the intended duration of therapy following desensitization can be maintained, as RDD effects a temporary state of tolerance, and premature cessation of the antibiotic may require re-desensitization before the completion of a course. This discussion focuses on intravenous rapid desensitization to antibiotics for immediate-type HSRs, and does not include slow oral desensitization regimens that have been described for delayed-type hypersensitivities to multiple antimicrobials, including trimethoprim/sulphamethoxazole, metronidazole, isoniazid, and antiretrovirals.

Experience with antibiotic desensitization, primarily with penicillins and cephalosporins, has accumulated over several decades following a case series describing penicillin desensitization in penicillin-sensitive pregnant women with syphilis [10]. Only immediate-type hypersensitivity reactions consistent with an IgE- and/or mast cell-mediated mechanism are considered amenable to desensitization. Such reactions include dermatologic (flushing, pruritus, urticaria, angiooedema), upper and lower respiratory tract (sneezing, sinus and nasal congestion, cough, dyspnoea, wheezing), gastrointestinal (abdominal pain, nausea, vomiting, diarrhoea), and cardiovascular manifestations (hypotension) during anaphylaxis. Patients with other reactions, including maculopapular rashes, fixed drug eruptions, Stevens–Johnson syndrome, toxic epidermal necrolysis, bullous erythema, drug reaction with eosinophilia and systemic symptoms (DRESS), transaminitis, acute interstitial nephritis, serum sickness, haemolytic anaemia, thrombocytopenia, or neutropenia, are not candidates for rapid intravenous desensitization.

The decision to embark upon antibiotic desensitization should be made in conjunction with an Infectious Diseases specialist to determine the relative advantages of first-line therapy over alternatives, the duration of treatment, and the goals of therapy. Evaluating the patient for desensitization requires taking a careful history to determine whether the initial reaction is consistent with a mast cell-/IgE-mediated hypersensitivity reaction, and assessing the patient's risk by determining the severity of and the time since the initial reaction. With the renewed availability of the major determinant penicilloyl polylysine, skin testing has once again become a validated component of the assessment of β-lactam allergies and can be particularly useful in patients with vague histories. Penicillin skin testing (extensively reviewed elsewhere [11–15]) provides a method of risk-stratifying patients with a history of reaction to penicillin. The penicillin skin test with the major and minor determinants of penicillin has a high negative predictive value [16, 17]. Following earlier data suggesting high rates of cross-sensitization to carbapenems in penicillin skin test-positive patients as measured by imipenem skin testing without challenge [18], a systematic imipenem challenge in penicillin skin test-positive patients has demonstrated very low true cross-reactivity between these classes [19]. While other studies have described the use of skin testing with non-penicillin antibiotics with increasing data for non-irritating concentrations, none of these testing protocols has been standardized and validated.

The literature on rapid desensitization to antibiotics largely consists of case reports, but there have been several case series in the last decade in cystic fibrosis patients [20–22], a population disproportionately affected by recurrent infections (particularly by *Pseudomonas aeruginosa*), antibiotic allergies, resistant organisms, and therefore in need of antibiotic desensitization. These studies provide data on the safety and feasibility of desensitization to various antibiotics, primarily β-lactams, in a high-risk population with poor lung function. All three studies, including one at our institution, were retrospective chart reviews of patients who underwent desensitization. Success rates ranged from 58% to 100%. Differences among the studies include patient age, nature of prior reactions, premedications, protocol including the starting dose and the rate of increase, definition of desensitization success, and threshold to stop desensitization. Mild to moderate reactions during desensitization did not preclude the completion of desensitizations and could be followed by full scheduled doses. Most patients required multiple desensitizations over time. In our case series, 15 patients completed 100% of 52 desensitizations, 45 without any reaction. Six patients experienced limited symptoms consistent with immediate-type hypersensitivity reactions. One patient had acute respiratory failure requiring intubation following ceftazidime desensitization, which was attributed to preexisting infection-related declining respiratory status, and later had uneventful desensitizations to ceftazidime. In another group of patients, nafcillin, penicillin, cefazolin, and ceftriaxone were among the antibiotics to which patients were successfully desensitized using our protocol [1].

Current recommendations for patients with a history of penicillin reactions who may require a penicillin or cephalosporin suggest penicillin skin testing with major and minor determinants when available [23]. Patients with negative skin testing should not require desensitization, and those with positive skin tests are recommended to avoid penicillins and cephalosporins, particularly

first-generation agents. If these medications are deemed necessary, desensitization to penicillins and cephalosporins may be quite useful in skin test-positive patients.

Although vancomycin is not the preferred agent for the treatment of β-lactam-susceptible infections, it is used in gram-positive infections with β-lactam resistance or in β-lactam-allergic patients. Its use continues to rise with the spread of community and hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) as well as in persistent and moderate-to-severe cases of *Clostridium difficile* colitis. Much more common than type I hypersensitivity reactions to vancomycin is the 'red man syndrome' (RMS), characterized by flushing, warmth, pruritus, and hypotension. RMS results from direct mast cell and basophil histamine release, can occur without prior exposure, and is not accompanied by an increase in tryptase [24]. While slowing the infusion rate usually ameliorates RMS, true hypersensitivity does not respond to this measure and may require desensitization. In addition to the several patients described in the three cystic fibrosis series, multiple series have been published on vancomycin desensitization regimens, both rapid (over hours) and slow (over days), and have been used successfully [25–29].

Fluoroquinolone hypersensitivity is less well understood, and there are few reports of ciprofloxacin desensitization in the literature [30]. Of the cystic fibrosis patient series described above, our series and the Boston Children's Hospital series each include a successful ciprofloxacin desensitization [21, 22], and the Prince Charles Hospital series includes a ciprofloxacin desensitization that was aborted because of a urticarial rash [20].

Hypersensitivity to trimethoprim/sulphamethoxazole most commonly presents as a delayed-type cutaneous eruption, and it is a frequent culprit in Stevens–Johnson syndrome. These toxicities are thought to be mediated by reactive metabolites that cannot be fully metabolized by glutathione stores [31]. Slow outpatient oral desensitizations are well described in patients with HIV/AIDS, who have a disproportionately high prevalence of hypersensitivity to this drug. We have limited experience with patients with the rarer immediate-type HSR, and have successfully performed rapid intravenous desensitizations in such patients [21].

Immediate HSRs to aminoglycosides are also quite rare, and aminoglycoside use is generally limited by vestibulo/ototoxicity and nephrotoxicity. We have described successful intravenous desensitization to tobramycin in a cystic fibrosis patient [21], and the Children's Hospital series includes a single failed gentamicin desensitization [22]. Tobramycin desensitizations via the intravenous and inhaled route have been described previously [32, 33].

Following desensitization, each scheduled full dose of the antibiotic must be administered in a timely fashion in order to prevent loss of the temporary desensitized state. As many penicillins have relatively short half-lives, careful consideration should be exercised when contemplating desensitization.

Rapid drug desensitization to chemotherapeutic agents: taxanes

Paclitaxel and docetaxel are cytotoxic drugs widely used in the treatment of ovarian, breast, non-small-cell lung, and other solid tumours. Hypersensitivity reactions to taxanes are common. In early trials of paclitaxel, up to 30% of patients developed acute infusion reactions. Premedication with antihistamines and glucocorticoids as well as slower infusion rates have reduced the rate of severe hypersensitivity reactions to $< 10\%$ [34–37]. Similarly, approximately 30% of patients receiving docetaxel without premedication developed acute hypersensitivity reactions, and premedication reduces this rate to $< 10\%$ [38].

Acute hypersensitivity reactions to taxanes are characterized by dyspnoea, urticaria, flushing, back pain, gastrointestinal symptoms, hypo- or hypertension, and erythematous rashes. Symptoms typically develop within the first few minutes of the infusion, and most often occur on the first or the second exposure to the drug [36, 39].

The characteristics of hypersensitivity reactions to paclitaxel and carboplatin are compared and contrasted in Fig. 8 [3, 40]. Both of these agents frequently cause cutaneous, cardiovascular, and gastrointestinal symptoms. However, while back pain is a frequent symptom in paclitaxel hypersensitivity reactions (36% of patients in this series), it is seldom seen in carboplatin reactions [3]. The mechanisms underlying these differences in presentation are not well understood.

Data on cross-reactivity of paclitaxel and docetaxel have been inconsistent. Previous small clinical trials (3–4 patients each) have described successful treatment with docetaxel following hypersensitivity reactions to paclitaxel [41, 42]. However, a more recent retrospective review found that nine out of 10 patients treated with docetaxel following a hypersensitivity reaction to paclitaxel also reacted to docetaxel, suggesting a higher rate of cross-reactivity [43].

The mechanisms of taxane infusion reactions are not completely understood and may be multifactorial. Proposed mechanisms include complement activation, direct mast cell and/or basophil activation, and IgE-mediated anaphylaxis [44]. Taxane reactions are unlikely to be due solely to an IgE response, because a majority of reactions (56% in one study) occur with the first exposure to paclitaxel, without the prior sensitization necessary for an IgE-mediated reaction [39]. There is evidence that both the taxane moiety itself and the vehicles in which these agents are solubilized can contribute to infusion reactions. Specifically, Paclitaxel is stabilized with Cremophor,

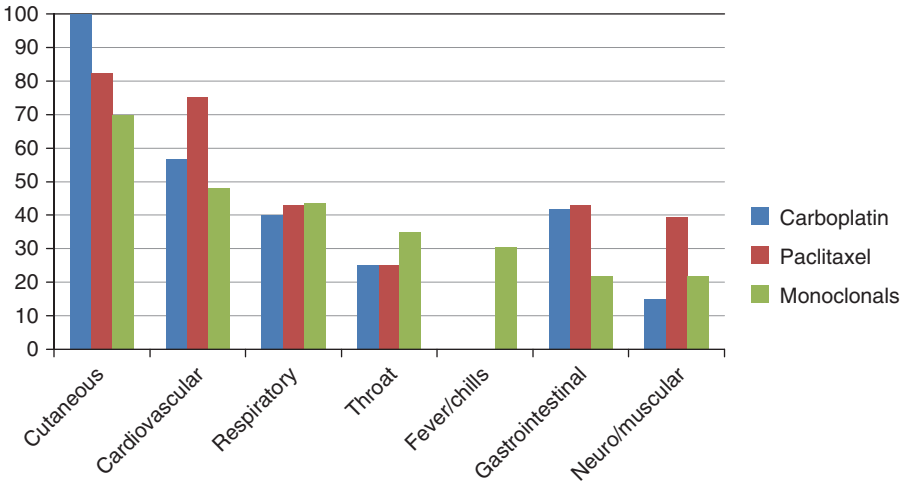

Fig. 8. Symptoms and signs of hypersensitivity reactions in 111 patients. (Adapted from Castells et al. [3] and Brennan et al. [40].)

which is derived from castor oil and is also used as the vehicle for other drugs, such as cyclosporine and vitamin K, which have been associated with similar adverse reactions [39, 45–48]. An albumin-based formulation of Paclitaxel, devoid of cremophor, has also been implicated in hypersensitivity reactions, providing further evidence for taxane moiety-based hypersensitivity reactions.

Desensitization to taxanes is generally well tolerated. In a series of 17 patients who underwent a total of 77 desensitizations to paclitaxel or docetaxel, 72 desensitizations occurred without reactions. Four patients had a total of five reactions during desensitization, all of which were much less severe than their original reactions. On the other hand, five patients who underwent re-challenge before desensitization experienced recurrent reactions, despite additional premedication and a reduced infusion rate [49]. In our series of 98 patients undergoing a total of 413 desensitizations to various chemotherapeutic agents, the majority of desensitizations had mild or no reactions, and most reactions occurred during the final, most concentrated solution, and specifically during the last step of the protocol [3].

Rapid drug desensitization to chemotherapeutic agents: platins

Platinum-containing compounds are some of the most biologically active cytotoxic drugs in the treatment of ovarian cancer, and have been used in the treatment of numerous malignancies since the 1970s. Cisplatin was the first to be used, but it was the relatively low toxicity profile of the second-generation carboplatin that is largely responsible for its increased popularity in the past decade [50]. The third-generation platinum derivative oxaliplatin is widely used for the treatment of metastatic colorectal cancer as well as other malignancies.

As the use of platinum-containing compounds has increased, so has the incidence of HSRs. The reported incidence of cisplatin hypersensitivity varies from 5 to 20%, carboplatin from 9% to 27%, oxaliplatin from 10% to 19% [51–53]. One salient feature of the platinum drugs is the requirement of repeated exposures before the onset of the hypersensitivity. Markman et al.[54] reported that of the 12% of patients receiving carboplatin who had an HSR, 50% of the initial episodes occurred during the eighth course. Other studies have corroborated these data as well. In one retrospective chart review at The University of Texas M.D. Anderson Center, the incidence of carboplatin HSR was 7.9% for ovarian cancer patients. In concordance with previously published data, those patients with carboplatin HSR on average received eight prior doses of carboplatin. Dividing the patients into those who had received $\geqslant 8$ cycles vs. those with <8, the incidence of HSR to carboplatin was 10.7% vs. 1.3%, respectively. Similarly, in ovarian cancer, the incidence of HSR in newly diagnosed patients was 2.1% vs. 17% among those with persistent/progressive disease and 12.6% in patients with recurrent disease [55]. Our group found that 40 out of 55 patients with carboplatin HSRs reacted between the 7th and the 10th exposure [3]. Cisplatin and oxaliplatin have similar characteristics in that reactions mostly occur between the 4th and the 8th course or after the 6th exposure, respectively [53].

The characteristics of HSRs to platinum agents vary widely. In the case of carboplatin, most patients develop cutaneous symptoms, notably palmar or facial flushing. However, half the patients may progress to moderate to severe reactions, and cardiac arrest and deaths have been reported [3]. In our report of 413 desensitizations, of the 60 patients who had carboplatin HSR, 100% had cutaneous symptoms, 57% had cardiovascular symptoms, 40% had respiratory symptoms, and 42% had gastrointestinal manifestations (see Fig. 8) [3].

Oxaliplatin HSRs are often similar to those seen in response to carboplatin and cisplatin, but there have been fewer reports of severe anaphylaxis. However, in contrast to carboplatin, respiratory symptoms are often the most common. Maindrault-Goebel and colleagues reported that of 42 patients with oxaliplatin HSR, 50% had respiratory symptoms including laryngeal spasms and hypoxaemia, whereas 40% of patients had cutaneous manifestations. Interestingly, they also reported three cases of a Gell and Coombs type II-mediated thrombocytopenia, and other authors have reported Gell and Coombs type III immune-complex-mediated symptoms of chronic urticaria, joint pain, and proteinuria associated with oxaliplatin. Idiosyncratic reactions to oxaliplatin, including cytokine release syndrome and pulmonary fibrosis, make adverse responses to oxaliplatin heterogeneous and unpredictable [53, 56, 57].

Being able to predict who is at a significant risk of a hypersensitivity reaction would allow for interventions before any adverse outcomes from platinum-containing compounds, without needlessly stopping or withholding medication from those at a low risk. It is clear that in the case of carboplatin, the risk of reaction increases sharply with the 8th exposure, which, in standard protocols, is generally the second cycle of the second treatment regimen. One group also noticed an association between the interval of a carboplatin-free period and the risk of HSR, especially a severe reaction. Schwartz and colleagues, in a study looking at 126 patients with HSR to carboplatin, noted that the risk of severe reactions was 47% if the platinum-free interval was >24 months, vs. only 6.5% if it was <12 months. All eight patients receiving their third carboplatin regimen showed severe reactions [58].

Using clinical characteristics to stratify risk has been attempted, generally with modest results. One group noted a statistically significant increase in the risk of reaction in patients with a past history of other allergic reactions to either medications or environmental allergens [59]. For oxaliplatin, Kim et al. [60] found that younger age, female sex, and the use of oxaliplatin as salvage therapy were all statistically significant risk factors for an HSR.

Skin testing has been used to predict platinum hypersensitivity, but methods vary widely from institution to institution. Markman and colleagues attempted to identify patients at risk of an HSR before a clinical reaction by performing prospective skin testing 1 h before the 7th cycle of carboplatin. They reported a negative predictive value of 98.5% (658/668). Of the 10 patients who reacted despite having negative skin testing, all of them had only mild cutaneous symptoms. There were 41 patients with positive skin tests, of whom seven were rechallenged, with six of the seven experiencing mild to moderate symptoms.

Our group skin tested 60 patients referred for previous HSRs to carboplatin. Of these, 53 were skin test positive. Of the seven with negative skin tests, two patients converted to positive skin tests after several infusions, one skin test was considered delayed positive, and four patients experienced hypersensitivity reactions during infusion [3].

Hesterberg and colleagues recently published a report of 38 women with carboplatin HSR who were skin tested and desensitized. Thirteen patients were skin test negative to carboplatin, and seven of those patients had reactions during a 'rapid desensitization protocol'. Interestingly, they found that when dividing the negative skin test group using the time from the HSR to skin testing, those with a recent history of HSR (<3 months) and negative skin tests did not react, whereas all seven of the reactors had a remote history of HSR (>9 months). Of note, this group uses a maximum carboplatin skin test dose of 3 mg/mL, while our group uses 10 mg/mL.

Once a patient has an allergic reaction to a platinum-containing compound or a positive skin test, the physician must then decide whether to attempt re-administration of the same agent, to change to a different platinum drug, or to desensitize the patient. The first two choices have produced mixed results, and deaths have been reported. Polyzos and colleagues reported a series of 32 patients rechallenged with carboplatin after HSRs. Four of the 20 patients with mild reactions again had erythema but were able to finish the medication infusions. However, 12 patients with initial severe reactions including hyper- or hypotension were unable to complete subsequent carboplatin infusions despite prophylaxis. Interestingly, in this report, four of the 12 were switched to cisplatin and tolerated infusions, but the true incidence of cross-reactivity among platinum-based chemotherapeutic agents is not known. Attempts to circumvent a reaction by switching to another platinum-based chemotherapeutic can be dangerous [61], as exemplified by Dizon et al. [62], who reported the death of one patient due to anaphylaxis in a series of seven patients switched from carboplatin to cisplatin.

A plethora of literature attests to the safety of desensitization as a way to allow a patient to continue carboplatin chemotherapy. However, there is variability in the success rates due to the fact that platinum desensitization is not standardized, and different institutions follow various methods and protocols. O'Cearbhaill and colleagues prophylactically converted 174 patients to an extended infusion schedule after the 8th cycle, with 1% of the dose administered in the first hour, 9% during the second hour, and 90% during the third hour. Of the 174 patients converted to this schedule, only six (3.4%) developed HSR vs. 111/533 (21%) of those remaining on a standard infusion protocol. An important caveat to these results is that this was a retrospective study, and so potential confounding factors such as premedications administered, prior drug allergy history, or number of infusions may not have been controlled for between the two groups [63].

Rapid drug desensitization to monoclonal antibodies

Monoclonal antibodies are rapidly becoming standard therapy in the treatment of a multitude of diseases. For the most part, they are well tolerated. However, a subset of patients experience HSRs following the administration of these drugs [64]. The symptoms of HSRs range from mild (fever, rash, pruritus) to severe, including severe life-threatening anaphylaxis [64].

The rates of HSRs that are clinically consistent with immediate hypersensitivity to monoclonal antibodies and other drugs considered in this section have been reported to be 5–10% for rituximab, 2–3% for infliximab, and 0.6–5% for trastuzumab [40]. Immediate HSRs have also been reported for omalizumab, natalizumab, basiliximab, abciximab, and cetuximab.

Cutaneous reactions were observed as a component of almost 70% of the initial reactions and were the most frequently observed type of reaction overall, followed by cardiovascular, respiratory, and throat tightness [64]. The intensity of reactions to monoclonal antibodies infusions is variable. Recent studies have reported that 26% of the initial reactions are mild, 48% are moderate, and 26% are severe [40]. Demographic studies have reported a markedly increased incidence of severe HSRs among patients living in the middle portion of the southeastern United States. One study recorded a rate of severe HSRs as high as 3% [65]. Follow-up studies of patients treated with cetuximab in clinical trials in Tennessee and North Carolina showed a rate of severe HSRs of 22% [66]. When the authors tried to identify associations between HSRs and risk factors including demographics, primary sites of cancer, and atopic history, only atopic history was significantly associated with the severe HSRs [66].

Patients with a history suggestive of a mast cell, possibly IgE-mediated HSR, should be skin tested with the offending agent as described previously [40]. HSRs are then classified as mild, moderate, or severe according to the classification system proposed by Brown [40]. Fever and/or chills, which are not included in the Brown classification, are classified as mild for subjective fever, or a measured $T < 38.0\,^{\circ}\mathrm{C}$. $T > 38.0\,^{\circ}\mathrm{C}$ was classified as a moderate reaction. The signs and symptoms of HSRs are classified as cutaneous (flushing, pruritus, urticaria, angiooedema), cardiovascular (chest pain, tachycardia, sense of impending doom, presyncope, syncope, and hypotension), respiratory (dyspnoea, wheezing, and oxygen desaturation), throat tightness, gastrointestinal (nausea, vomiting, diarrhoea, and abdominal pain), neurological/muscular (vision disturbances, back and neck pain, and numbness/weakness), and fever/chills [40].

Protocols for most monoclonal antibodies are generated using the same principles as previously discussed above. Despite its general success, some patients experience HSRs during RDD. In general, these reactions are less intense than the patient's original reaction. Treatment of such HSRs is aimed at blocking mast cell mediators including histamine, prostaglandins, and leukotrienes [40]. In the event of a reaction during RDD, the infusion is promptly held. Further reactions are managed clinically based on the algorithm in Fig. 9.

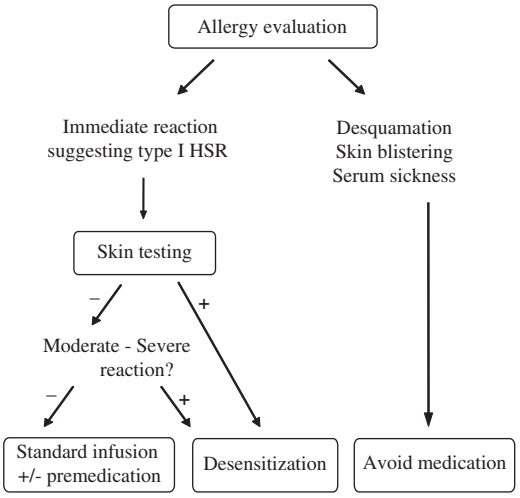

Fig. 9. Algorithm for the assessment and treatment of patients with HRS (from Brennan et al. [40]).

Recent data show reactions rates of 29% during monoclonal antibody desensitization, with 90% of these reactions being mild [40]. In the small percent of cases that had a severe reaction, all patients retrospectively had positive skin testing to the agent administered [40] As expected, cutaneous reactions were the most frequent type of reaction observed during desensitization. It was also observed that 70% of reactions during desensitization occurred during the 12th and the final step using our standard 12-step protocol [40]. Delayed reactions have been reported but to date these reactions have all been mild [40].

Conclusions

Although the molecular basis of RDD is not completely understood, the protocols have been remarkably successful. Over the past 10 years, more than 99.9% of nearly 800 patients have received the full dose of their first-line medication in thousands of desensitizations, and there have been no deaths from HSRs [3]. These safety and efficacy outcomes provide grounds for the continued and expanded use of this approach to RDD for all patients for whom a drug hypersensitivity would prevent the administration of first-line pharmacologic therapy. Using a standardized BWH 12-step protocol, we have been able to treat hundreds of patients with infections, cancer, and inflammatory conditions, providing improved quality of life and increased survival rates. This desensitization protocol is an innovative and useful tool for all medical specialties when applied under the supervision of trained allergists.

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TITLE: Desensitization for Hypersensitivity Reactions to Medications

AUTHORS: Maria del Carmen Sancho, Rebecca Breslow, David Sloane, and Mariana Castells

Introduction

Adverse reactions to drugs are increasingly recognized as important contributors to disease as well as impediments to the best treatment of various maladies, including dermatological, infectious, autoimmune, and neoplastic disorders. With the development of novel pharmacologic agents and the evolution of personalized treatments based on pharmacogenetic profiling, clinicians must decide which agent is the best for a particular patient with a given disease. Biological agents have greatly improved the treatment of chronic inflammatory diseases and malignancies while limiting some medication associated toxicities. Because of better outcomes, longer patient survival, and extended treatment courses, patients are exposed to drugs more frequently and for longer time periods, increasing the risk of sensitization to medications. The frequency of adverse drug reactions has increased in the last 10 years. Because of the severity of some reactions and the fear of inducing a potentially lethal reaction in highly sensitized patients, first line treatments are sometimes abandoned, relegating hypersensitive patients to secondary, less effective, therapy. Some of these reactions are mast cell mediated hypersensitivity reactions (HSRs), a subset of which occur through an IgE dependent mechanism, and are thus true allergies. Others involve mast cells but an IgE mechanism cannot be demonstrated. Rapid drug desensitization (RDD) is a technique that induces temporary tolerance to a drug, allowing a medication allergic patient to receive the optimal agent for his or her disease. Through RDD, patients with IgE and non-IgE HSRs can safely be administered important medications while minimizing or completely inhibiting adverse reactions.

General Principles and Proposed Mechanisms of Rapid Drug Desensitization

Exposure of IgE-sensitized patients to medication can cause the sudden systemic release of inflammatory mediators from activated mast cells, leading to anaphylaxis [1, 2] and medication avoidance, while effective for circumventing an HSR, may lead to significant morbidity and mortality from suboptimal treatment of disease. RDD is a process by which mast cells are rendered hypo-responsive to an agent that, when administered in typical fashion, induces mast cell activation and degranulation. RDD provides temporary tolerization for drug hypersensitive patients, protecting them from anaphylaxis. Desensitization protocols have been developed to help deliver full therapeutic doses of drug allergens, in an incremental, stepwise fashion without eliciting life-threatening symptoms [3-5]. Most IgE-sensitized patients present with a positive skin test to the medication, indicating that mast cells (likely through drug specific IgE) are the main cells responsible for these reactions. After rapid desensitization, specific skin test reactivity is abolished, implying a complete inhibition of the mechanisms that induced mast cell activation [6].

Mast cells activated by antigen crosslinking of IgE-bound FcεRI receptors display aggregation of these receptors, recruitment and activation of target molecules, calcium mobilization, degranulation, arachidonic acid metabolism, and cytokine and chemokine gene transcription [7, 8]. RDD then induces mast cell tolerization to antigen via Internalization of FcεRI. This may occur through progressive crosslinking at low antigen concentration, sub-threshold depletion of mediators, and depletion of activating signal transduction components such as syk kinase, all of which have all been postulated as mechanisms for cellular unresponsiveness to specific activating doses of allergen [9, 10].

To test these hypotheses, we and others developed a reproducible *in vitro* model of antigen specific, rapid mast cell/IgE desensitization in the presence of physiologic levels of calcium (Figure 1). Increasing doses of antigen delivered at fixed time intervals induced a highly specific and prolonged hypo-responsiveness to triggering doses of the desensitizing antigen. Mast cells desensitized to DNP or OVA antigens demonstrated almost complete inhibition of β -hexosaminidase and pre-formed TNF-α release, calcium flux, and arachidonic acid metabolism, suggesting a complete abolition of the acute phase of mast cell activation and demonstrating that the subclinical release of mediators was unlikely during human desensitizations. Desensitized mast cells did not release significant amounts of newly generated IL-6 or TNF-α, confirming that during rapid desensitization patients were not at risk for a delayed reaction due to the lack of late phase mediators generation.

When mast cells were sensitized to both DNP and OVA antigens, DNP-desensitized cells responded fully to OVA and vice versa, proving antigen specificity and providing evidence that the activating signal transduction pathways are intact for a second allergen. Therefore, the hypothesis that activating signaling molecules are exhausted during rapid desensitization is not supported. Importantly, antigen-specific IgE bound to the alpha chain of FcεRI remained at the membrane level after rapid desensitization, indicating that the lack of reactivity during desensitization was not due to the disappearance of surface IgE and FceRI when bound to small doses of antigen (Figure 2). Thus, the biochemical mechanism(s) by which RDD induces mast cell tolerance are still unclear. However, this *in vitro* model provided an optimal dose-time relationship, leading to almost complete abrogation of early- and late-phase activation events, providing a basis for a modified human rapid desensitization protocol that has been used successfully in hundreds of desensitizations, illustrating the profound inhibition of acute and delayed mast cell responses and the protection against anaphylactic reactions [4, 5].

Clinical Rapid Desensitization: Protocols and Agents

The BWH Desensitization Program devised a 12- to 20-step standard protocol based on the above *in vitro* mouse mast cell model, in which unresponsiveness to a triggering antigen dose was achieved by delivering doubling doses of antigen at fixed time intervals starting at 1/1000 dilution of the final dose [11]. The most commonly used protocol has 12-steps, using three ten-fold diluted solutions at escalating rates (Figure 3). Patients who have had severe anaphylactic reactions to the given agent, or who have reacted early in the standard 12-step desensitization may experience fewer symptoms if desensitized using a 16-step protocol, which adds another bag containing a 1/10000 dilution of the full dose. The use of a 16-step (4 bags) or 20-step (5 bags) protocol is reserved for high risk patients (see below). Drug desensitization is more than a protocol; it is an approach to specialized patient care. It thus starts with an allergy evaluation of the patient, including an in depth historical analysis of the patient's HSR, skin testing when available, design and testing of an initial desensitization protocol, and adjustment of this protocol in an iterative fashion based on the patient's response.

Below, we summarize our experience with rapid desensitization to four different classes of drugs: antibiotics, taxane chemotherapy agents, platinum-based chemotherapeutic agents, and monoclonal antibodies and other miscellaneous medications.

Rapid Drug Desensitization (RDD) to Antibiotics

Despite a wide selection of antibiotics available for treatment of inpatient and outpatient infections, a single antibiotic often emerges as the preferred choice in a given situation. If the chosen antibiotic is one to which the patient has a history of HSR, but drug resistance, prohibitive intolerances, limited bactericidal or bacteriostatic activity, and poor bioavailability of alternatives pose a risk of uncontrolled infection, desensitization is the best course. Unlike

chemotherapy and monoclonal antibodies, antibiotics are usually administered in doses scheduled 6 to 24 hours apart for several days to weeks. This discussion focuses on intravenous rapid desensitization to antibiotics for immediate type HSRs, and does not include slow oral desensitization regimens that have been described for delayed-type hypersensitivities to multiple antimicrobials, including trimethoprim/sulfamethoxazole, metronidazole, isoniazid, and antiretrovirals.

Experience with antibiotic desensitization, primarily with penicillins and cephalosporins, has accumulated over several decades following a case series describing penicillin desensitization in penicillin-sensitive pregnant women with syphilis[12]. Only immediate-type hypersensitivity reactions consistent with an IgE- and/or mast cell-mediated mechanism are considered amenable to desensitization. Such reactions include dermatologic (flushing, pruritus, urticaria, angioedema), upper and lower respiratory tract (sneezing, sinus and nasal congestion, cough, dyspnea, wheezing), gastrointestinal (abdominal pain, nausea, vomiting, diarrhea), and cardiovascular manifestations (hypotension) during anaphylaxis. Patients with other reactions, including maculopapular rashes, fixed drug eruptions, Stevens-Johnson syndrome, toxic epidermal necrolysis, bullous erythema, drug reaction with eosinophilia and systemic symptoms (DRESS), transaminitis, acute interstitial nephritis, serum sickness, hemolytic anemia, thrombocytopenia, or neutropenia, are not candidates for rapid intravenous desensitization.

Once an evaluation of the patient determines that the initial reaction is consistent with a mast cell-/IgE-mediated hypersensitivity reaction, determining the severity of and the time since the initial reaction makes an assessment of the patient's risk. Penicillin skin testing (reviewed elsewhere [13-17]) helps in risk-stratifying patients with a history of reaction to penicillin. Such testing has high sensitivity and specificity in estimating the likelihood of reacting to penicillin derivatives and moderate utility in assessing the risk for reacting to cephalosporins, especially

first generation cephalosporins[18, 19]. Following earlier data suggesting high rates of cross sensitization to carbapenems in penicillin skin test positive patients as measured by imipenem skin testing without challenge[20], systematic imipenem challenge in penicillin skin test positive patients has demonstrated very low true cross reactivity between these classes[21].. While other studies have described the use of skin testing with non-penicillin antibiotics with increasing data for nonirritating concentrations, none of these testing protocols has been standardized and validated.

The literature on rapid desensitization to antibiotics is largely case reports, but several case series in the last decade in cystic fibrosis patients[22-24], a population disproportionately affected by recurrent infections (particularly by *Pseudomonas aeruginosa*), antibiotic allergies, resistant organisms, and therefore in need of antibiotic desensitization, have established the safety and efficacy of desensitization to various antibiotics. Three studies, including one at our institution, were retrospective chart reviews of patients who underwent desensitization. Success rates ranged from 58% to 100%. Mild to moderate reactions during desensitization did not preclude completion of desensitizations, and could be followed by full scheduled doses. Most patients required multiple desensitizations over time. In our case series, 15 patients completed 100% of 52 desensitizations, 45 without any reaction. Six patients experienced limited symptoms consistent with immediate type hypersensitivity reactions. One patient had acute respiratory failure requiring intubation following ceftazidime desensitization, which was attributed to preexisting infection-related declining respiratory status, and later had uneventful desensitizations to ceftazidime. In another group of patients, nafcillin, penicillin, cefazolin, and ceftriaxone were among the antibiotics to which patients were successfully desensitized using our protocol[3].

Current recommendations for patients with a history of penicillin reactions who may require a penicillin or cephalosporin suggest penicillin skin testing with major and minor determinants when available[25]. Patients with negative skin testing should not require desensitization, and those with positive skin tests are recommended to avoid penicillins and cephalosporins, particularly first generation agents. If these medications are deemed necessary, desensitization to penicillins and cephalosporins is useful.

Vancomycin is often used in infections with beta-lactam resistant gram-positive organisms or in beta-lactam allergic patients. Its use continues to rise with the spread of community and hospital acquired methicillin-resistant *Staphylococcus aureus* (MRSA) as well as in persistent and moderate-to-severe cases of *Clostridium difficile* colitis. Much more common than type I hypersensitivity reactions to vancomycin is “red man syndrome” (RMS), characterized by flushing, warmth, pruritus, and hypotension. RMS results from direct mast cell and basophil histamine release, can occur without prior exposure, and is not accompanied by an increase in tryptase[26]. While slowing the infusion rate usually ameliorates RMS, true hypersensitivity does not respond to this measure and may require desensitization. Multiple series have been published on successful vancomycin desensitization regimens, both rapid (over hours) and slow (over days) [27-31].

A few reports of ciprofloxacin desensitization exist in the literature[32]. Of the cystic fibrosis patient series described above, our series and that from The Children’s Hospital in Boston each include a successful ciprofloxacin desensitization[23, 24]. The Prince Charles Hospital series includes a ciprofloxacin desensitization that was aborted because of an urticarial rash[22].

Hypersensitivity to trimethoprim/sulfamethoxazole most commonly presents as a delayed type cutaneous eruption, and it is a frequent culprit in Stevens-Johnson syndrome. These toxicities are thought to be mediated by reactive metabolites that cannot be fully metabolized by

glutathione stores[33]. Slow outpatient oral desensitizations are well described in patients with HIV/AIDS, who have a disproportionately high prevalence of hypersensitivity to this drug. We have limited experience with patients with the rarer immediate type HSR, and have successfully performed rapid intravenous desensitizations in such patients[23].

Immediate HSRs to aminoglycosides are also relatively rare. We have described successful intravenous desensitization to tobramycin in a cystic fibrosis patient[23], and the Children's Hospital of Boston series includes a single failed gentamicin desensitization[24]. Tobramycin desensitizations via the intravenous and inhaled route have been described previously [34, 35].

Following desensitization, each scheduled full dose of the antibiotic must be administered in a timely fashion in order to prevent loss of the temporary desensitized state.

Rapid Drug Desensitization (RDD) to Chemotherapeutic Agents: Taxanes

Paclitaxel and docetaxel are widely used in the treatment of ovarian, breast, non-small cell lung, and other solid tumors. Hypersensitivity reactions to these taxanes are common: in early trials of paclitaxel, up to 30% of patients developed acute infusion reactions. Premedication with antihistamines and glucocorticoids as well as slower infusion rates have reduced the rate of severe hypersensitivity reactions to less than 10% [36-39]. Similarly, approximately 30% of patients receiving docetaxel without premedication developed acute hypersensitivity reactions, and premedication reduces this rate to less than 10% [40].

Acute hypersensitivity reactions to taxanes are characterized by dyspnea, urticaria, flushing, back or chest severe pain, gastrointestinal symptoms, hypo- or hypertension, and erythematous rashes. Symptoms typically develop within the first few minutes of the infusion, and most often occur on the first or second exposure to the drug [38, 41]. The mechanisms of taxane infusion reactions are not completely understood and may be multifactorial. Proposed mechanisms

include complement activation, direct mast cell and/or basophil activation, and IgE-mediated anaphylaxis [42]. Taxane reactions are unlikely to be due solely to an IgE response, because a majority of reactions (56% in one study) occur with the first exposure to paclitaxel, without the prior sensitization necessary for an IgE-mediated reaction [41]. There is evidence that both the taxane moiety itself and the vehicles in which these agents are solubilized can contribute to infusion reactions. Specifically, Paclitaxel is stabilized with Cremophor, which is derived from castor oil and is also used as the vehicle for other drugs, such as cyclosporine and vitamin K, which have been associated with similar adverse reactions [41, 43-46]. An albumin–based formulation of Paclitaxel, devoid of cremophor, has also been implicated in hypersensitivity reactions, providing further evidence for taxane moiety-based hypersensitivity reactions.

Desensitization to taxanes is generally well tolerated. In a series of 17 patients who underwent a total of 77 desensitization to paclitaxel or docetaxel, 72 desensitizations occurred without reactions. Four patients had a total of 5 reactions during desensitization, all of which were much less severe than their original reactions. On the other hand, 5 patients who underwent re-challenge (i.e., re-administration of the culprit taxane by regular infusion) prior to desensitization experienced recurrent reactions, despite additional premedication and a reduced infusion rate [47]. In our series of 98 patients undergoing a total of 413 desensitizations to various chemotherapeutic agents, the majority of desensitizations had mild or no reactions, and most reactions occurred during the final, most concentrated solution, and specifically during the last step of the protocol [5].

Rapid Drug Desensitization (RDD) to Chemotherapeutic Agents: Platins

The platinum containing compounds are extensively employed in the treatment of ovarian cancer and other malignancies. Cisplatin was the first to be used, but it was the relatively low toxicity profile of the second generation carboplatin that is largely responsible for its increased

popularity in the past decade [48]. The third generation platinum derivative oxaliplatin is widely administered for the treatment of metastatic colorectal cancer. As the use of platinum containing compounds has increased, so has the incidence of HSRs: cisplatin hypersensitivity varies from 5-20%, carboplatin from 9% to 27%, and oxaliplatin from 10-19% [49-51]. Unlike the situation with taxanes, repeated exposures are typically required prior to the onset of hypersensitivity to platins. In one study, 50% of the initial HSRs to a platin occurred during the eighth course [52]. Likewise, we found that 40 out of 55 patients with carboplatin HSRs reacted between the 7^{th} to 10^{th} exposure [5]. Cisplatin and oxaliplatin have similar characteristics in that reactions mostly occur between the 4^{th} and 8^{th} course or after the 6^{th} exposure, respectively [51].

The characteristics of HSRs to platinum agents vary widely. In the case of carboplatin, most patients develop cutaneous symptoms; notably palmar or facial flushing. However, half of patients may progress to moderate to severe reactions, and cardiac arrest and deaths have been reported [5]. In our report of 413 desensitizations, of the 60 patients who had carboplatin HSR, 100% had cutaneous symptoms, 57% had cardiovascular symptoms, 40% had respiratory symptoms, and 42% had gastrointestinal manifestations [5].

Oxaliplatin HSRs are often similar to those seen in response to carboplatin and cisplatin, but there have been fewer reports of severe anaphylaxis. However, in contrast to carboplatin, respiratory symptoms are common, and other reactions such as Gell and Coombs type II mediated thrombocytopenia and Gell and Coombs type III immune-complex mediated symptoms of chronic urticaria, joint pain, and proteinuria associated, have been reported in response to oxaliplatin. Idiosyncratic reactions to oxaliplatin, including cytokine release syndrome and pulmonary fibrosis, make adverse responses to oxaliplatin heterogeneous and unpredictable [51, 53, 54].

There is a well-recognized association between the interval of carboplatin free period and

the risk of HSR, especially a severe reaction. Schwartz *et al.* in a study looking at 126 patients with HSR to carboplatin noted that the risk of severe reactions was 47% if the platinum free interval was >24 months, versus only 6.5% if it was <12 months. All 8 patients receiving their third carboplatin regimen had severe reactions [55].

Skin testing has been used to predict platinum hypersensitivity, but methods vary widely from institution to institution. Our group skin tested 60 patients referred for previous HSRs to carboplatin. Of these, 53 were skin test positive. Of the 7 with negative skin tests, 2 patients converted to positive skin tests after several infusions, one skin test was considered delayed positive, and 4 patients experienced hypersensitivity reactions during infusion [5]. Hesterberg *et al.* recently published a report of 38 women with carboplatin HSR who were skin tested and desensitized. Thirteen patients were skin test negative to carboplatin, and 7 of those patients had reactions during a "rapid desensitization protocol". Interestingly, they found that when dividing the negative skin test group using the time from the HSR to skin testing, those with recent history of HSR (<3 mo) and negative skin tests did not react, whereas all 7 of the reactors had remote history of HSR (>9 mo). Of note, this group uses a maximum carboplatin skin test dose of 3 mg/ml, while our group uses 10mg/ml.

Patients hypersensitive to a platinum-containing compound or with a positive skin test may be treated by an attempt to re-administer the same agent, or the decision may be to change to a different platinum drug, or to be desensitized. The first two choices have produced mixed results, and deaths have been reported. Polyzos *et al.* reported a series of 32 patients rechallenged with carboplatin after HSRs. Four of the 20 patients with mild reactions again had erythema but were able to finish the medication infusions. However, 12 patients with initial severe reactions including hyper- or hypotension were unable to complete subsequent carboplatin infusions despite prophylaxis. Interestingly in this report, 4 of the 12 were switched

to cisplatin and tolerated infusions, but the true incidence of cross reactivity among platinum-based chemotherapeutic agents is not known. Attempts to circumvent a reaction by switching to another platinum-based chemotherapeutic can be dangerous [56], as exemplified by Dizon *et al.* who reported the death of one patient due to anaphylaxis in a series of 7 patients switched from carboplatin to cisplatin [57].

Desensitization has proven to be a safe and effective way to allow a patient to continue carboplatin chemotherapy (see below). Variability in the success rates of desensitization is believed to be due to heterogeneity of methods and protocols.

Rapid Drug Desensitization (RDD) to Monoclonal Antibodies

Monoclonal antibodies are generally well tolerated treatments for a broad array of diseases, including malignancies and chronic inflammatory conditions. However, a subset of patients experience HSRs following administration of these drugs [58]. Symptoms of such HSRs range from mild (fever, rash, pruritus) to severe, including severe life threatening anaphylaxis [58].

The rates of HSRs clinically consistent with immediate hypersensitivity to specific monoclonal antibodies have been reported to be 5-10% for rituximab, 2-3% for infliximab, and 0.6-5% for trastuzumab [59]. Immediate HSRs have also been reported for omalizumab, natalizumab, basiliximab, abciximab, and cetuximab. Almost 70% of initial HSRs to monoclonal antibodies include a cutaneous component, the most frequently observed type of reaction overall, followed by cardiovascular, respiratory, and throat tightness [58]. The intensity of reactions to monoclonal antibodies infusions is variable. Recent studies have reported that 26% of initial reactions are mild, 48% are moderate, and 26% are severe [59].

Patients with a history suggestive of a mast cell, possibly IgE-mediated HSR should be skin tested with the offending agent as previously described be Lee 2004 [6]. HSRs are then

classified as mild, moderate, or severe according to the classification system proposed by Brown [61] . Signs and symptoms of HSRs are classified as cutaneous (flushing, pruritus, urticaria, angioedema), cardiovascular (chest pain, tachycardia, sense of impending doom, presyncope, syncope, and hypotension), respiratory (dyspnea, wheezing, and oxygen desaturation), throat tightness, gastrointestinal (nausea, vomiting, diarrhea, and abdominal pain), neurological/muscular (vision disturbances, back and neck pain, and numbness/weakness), and fever/chills [61]. Protocols for most monoclonal antibodies are generated using the same principles as previously discussed above. Despite its general success, some patients experience HSRs during rapid drug desensitization. In general, these reactions are less intense than the patient's original reaction. Treatment of such HSRs is aimed at blocking mast cell mediators including histamine, prostaglandins, and leukotrienes [59]. In the event of a reaction during rapid drug desensitization, the infusion is promptly held and the reaction treated. Once the reaction resolves, the protocol can almost always be resumed and completed.

Overall Safety and Efficacy

In 2008, our group reported the largest case series of rapid desensitizations, in which 98 patients with HSRs to chemotherapy underwent 413 desensitizations [5]. In this series, 67% of desensitizations proceeded without HSR, and 27% had only mild reactions (classified as absence of chest pain, changes in blood pressure, dyspnea, oxygen desaturation or throat tightness), even though 77% of patients had experienced a severe initial HSR. The remaining 6% of desensitizations were characterized by severe HSRs, however, epinephrine was only administered during one desensitization, and there were no transfers to a more acute-care setting, intubations, or deaths. All patients in the case series were able to receive their full target dose.

We subsequently published a case series of 105 desensitizations to monoclonal antibodies in 23 patients [59]. Seventy four percent of the initial HSRs were moderate to severe. During desensitization, reactions were observed in 29% of desensitizations and 90% of these were mild. Antibiotic desensitization using our protocol is also exceedingly safe [23]: in our case series of 52 antibiotic desensitizations in 15 patients with cystic fibrosis (and a mean FEV1 of 44.1% of predicted), 96.2% of desensitizations were completed without severe adverse events. One patient did develop severe acute respiratory failure requiring intubation, however, this was felt to be secondary to worsening pulmonary infection and not a manifestation of a severe HSR during his desensitizations. All desensitizations in this series were completed, suggesting that even markedly impaired baseline lung function is not a contraindication to rapid desensitization.

Treatment of Reactions During Desensitization

In our experience, reactions during desensitization manifest as a wide range of symptoms characteristic of HSRs [59]. Cutaneous reactions may include flushing, pruritis, urticaria and angioedema. More severe reactions may encompass cardiovascular manifestations, such as chest pain, tachycardia, a sense of impending doom, presyncope, syncope and hypotension, as well as respiratory symptoms, including sneezing, nasal congestion, dyspnea, coughing, wheezing, and oxygen desaturation. Severe reactions may also be characterized by throat tightness or gastrointestinal complaints, including nausea, vomiting, diarrhea and abdominal pain. Less common signs and symptoms may include neuromuscular symptoms, such as visual changes, back and neck pain, and numbness/weakness, or, in some cases, fever and chills.

In our 2008 case series of 413 desensitizations in 98 patients, there were a total of 180 reactions, all of which subsided when the infusion was paused and treated appropriately [5]. The majority of reactions (75%) occurred during infusion of solution 3, and 51% of reactions

occurred during Step 12 of the desensitization protocol. In our monoclonal antibody case series, in which a similar rate of reactions was reported (29%), cutaneous reactions were the most common and, again, the majority of reactions (70%) occurred during Step 12. Our approach to treating reactions during desensitization is aimed at blocking local and systemic effects of mast cell mediators, including histamine, prostaglandins, and leukotrienes [59].

At our institution, all reactions during desensitization are treated by pausing the infusion and administering either diphenhydramine or hydoxyzine (25-50 mg administered intravenously) and/or ranitidine (50 mg intravenously). For severe reactions, we most commonly use methylprednisolone sodium succinate (0.5 mg/kg administered intravenously). We keep epinephrine, 0.3 mL (1 mg/mL) at the bedside. On resolution of the reaction, we restart the protocol from the step at which it had been paused. Patients who experience reactions are then presented and discussed at a weekly meeting of the physicians in our department who perform rapid desensitizations.

We have adopted a two-pronged approach to protocol modification for subsequent desensitizations for patients who react during a prior desensitization [59]. The first component includes administration of additional premedications prior to the start of the protocol or between specific steps during desensitization. Most commonly, these are H1 and sometimes H2 blockers and/or methylprednisolone. These are generally added at least one full step before the point at which the reaction occurred. The second component of our protocol modification involves adding or lengthening steps before the step at which a reaction occurred. This second component is used only when a patient reacts despite additional premedications. By using this approach, we have been able to markedly reduce the rate of reactions over multiple successive desensitizations [5, 59].

Unfortunately, there remains a subset of patients who continue to react during desensitization despite protocol modification and addition of high-dose histamine receptor blockade and corticosteroids. In another case series, we prophylactically treated these patients with oral acetylsalicylic acid, 325 mg and oral montelukast 10 mg, and were able to successfully treat those patients with refractory mast cell mediator-related symptoms during rapid desensitization [62]. In this study, 78 desensitizations were performed in 14 patients with HSR to platinum chemotherapy that had cutaneous symptoms, many also with associated systemic reactions, during rapid desensitization. Pretreatment with ASA and montelukast 2 days before and on the day of RDD allowed 86% of the patients to tolerate subsequent desensitizations with a less severe or no HSR (Figure 4). Interestingly, only 62% of patients in a control group that received adjunctive methylprednisolone premedication were able to tolerate further desensitizations with a less severe or with no reaction. The greatest benefit of ASA/montelukast pretreatment was seen in patients with skin and respiratory symptoms, suggesting a dominant role for prostaglandins and leukotrienes in these manifestations of HSR to platinum chemotherapies. We have subsequently also treated patients with only one dose of ASA/montelukast sixty minutes prior to RDD, and have expanded this treatment for use during monoclonal antibody and antibiotic desensitization, and have successfully blocked refractory skin and systemic reactions using this regimen [23, 59].

Conclusions:

At our institution, we have had success using an intravenous RDD protocol to treat HSRs to a wide range of medications, including chemotherapeutics, monoclonal antibodies, and antibiotics. Over the past 10 years, more than 99.9% of nearly 800 patients have received the full dose of their first line medication in thousands of desensitizations to a wide variety of agents

in each of these three classes, and there have been no deaths. Although the molecular basis of RDD remains incompletely understood, an in vitro mast cell model has provided evidence of profound inhibitory mechanisms of mast cell activation during desensitization, which correlates with the remarkable success of the desensitization protocols when used by trained allergists. These safety and efficacy outcomes provide grounds for the continued and expanded use of this RDD approach for all patients for whom a drug hypersensitivity would prevent the administration of first line pharmacologic therapy.

Figure 1. Rapid desensitization impairs early- and late-phase mast cell activation responses . (**A**) % β-hexosaminidase release after desensitization (DNPDes or OVADes) or DNP-HSA or OVA challenge (1 ng DNP or 10 ng OVA) and negative control HSA. (**B**) Calcium flux when 1 ng DNP-HSA is added to cells treated as indicated. (**C**) RP-HPLC analysis of arachidonic acid products (LTC4 and LTB4) in supernatants of cells treated as indicated. (**D**) TNF-α and IL-6 secretion from mast cells during the early (30 min) and late (4 hours) phase of antigen activation and during rapid desensitization. **Adapted from Sancho-Serra et al [63]**

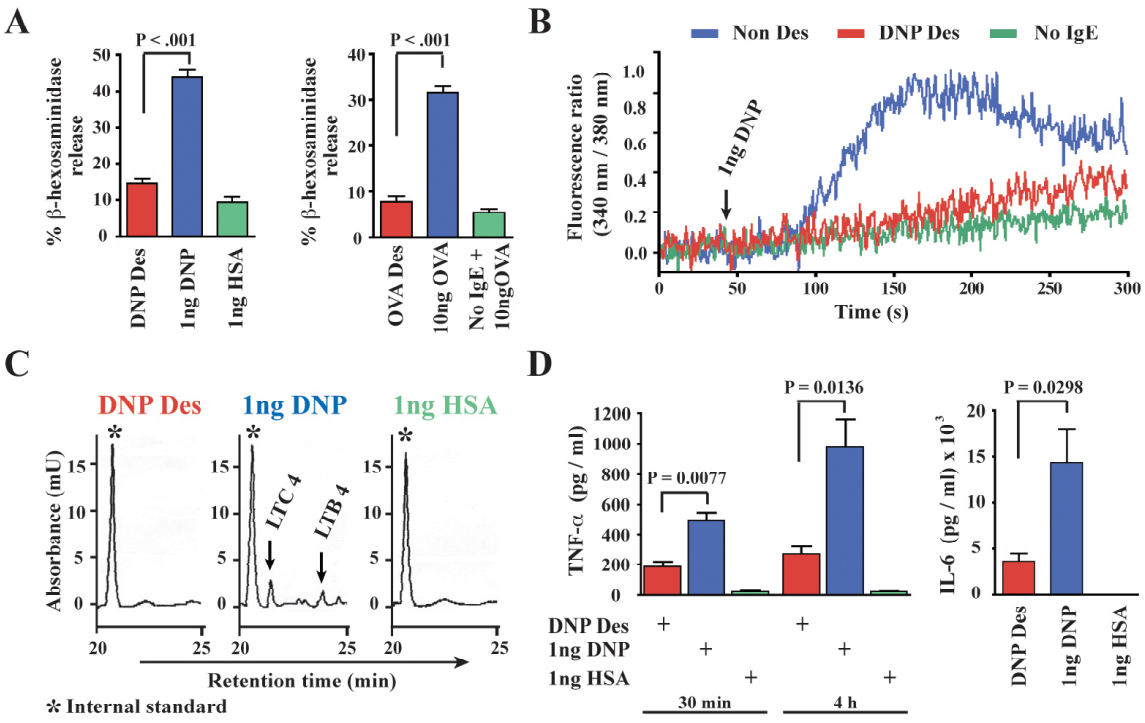


Figure 2. Mast Cell Antigen/IgE/FcεRI complex internalization is inhibited during rapid desensitization but does not impair specific activation. (**A**) Cells were treated as indicated and the FcεRIα and IgE surface expression were analyzed by flow cytometry. The blue line shows the internalization of the Antigen/IgE / **FcεRI during activation** as opposed to the red line in which no internalization occurs during desensitizations. (**B**) Confocal microscopy of cells treated as indicated. Cells activated with OVA (second panel) presented intracellular green fluorescence indicative of antigen internalization while desensitized cells (first panel) presented little internalization. Cells desensitized to OVA responded to DNP (fourth panel) indicating that the desensitization process if highly specific. **Adapted from Sancho-Serra et al** [63]

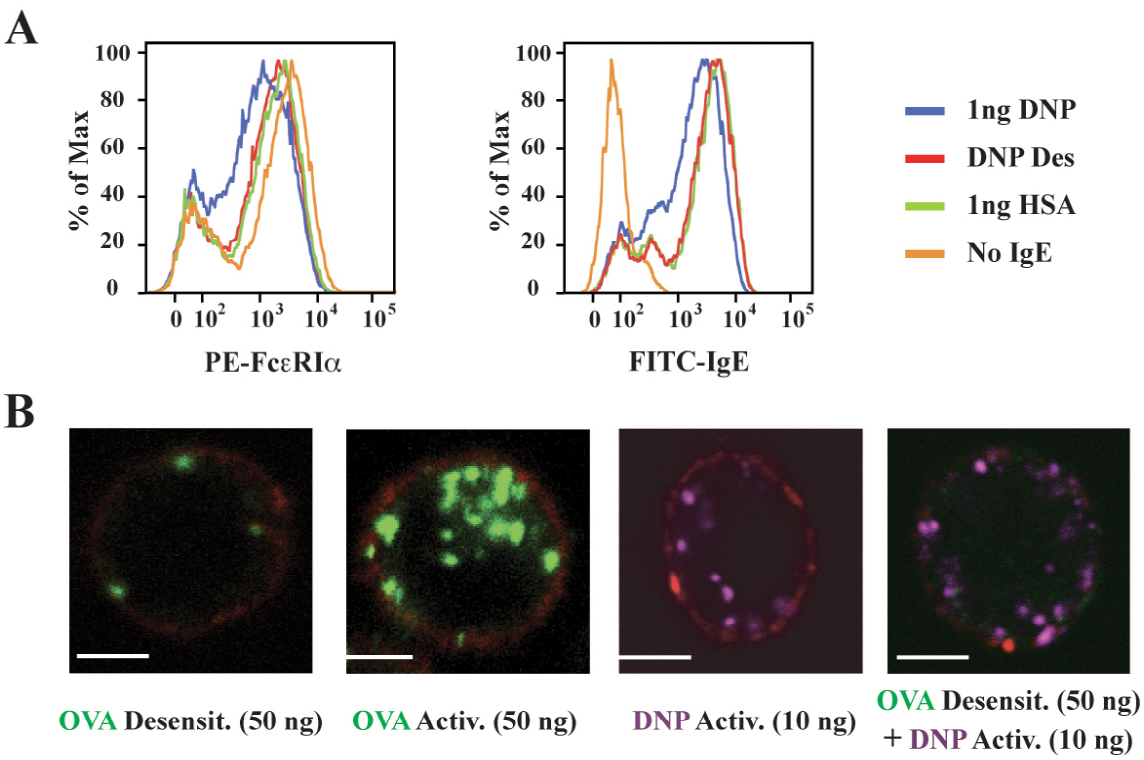


Figure 3: The standard 12 step, 3 bag desensitization protocol from the Brigham and Women's Hospital Rapid Drug Desensitization Program. Adapted from Castells et al. JACI 2008.

Name of medication:			**infliximab**		
Target Dose (mg)					800.0
Standard volume per bag (ml)					250
Final rate of infusion (ml/hr)					80
Calculated final concentration (mg/ml)					3.2
Standard time of infusion (minutes)					187.5
					Total mg per bag
Solution 1	250	ml of	0.032	mg/ml	8.000
Solution 2	250	ml of	0.320	mg/ml	80.000
Solution 3	250	ml of	3.175	mg/ml	793.704

*** PLEASE NOTE *** The total volume and dose dispensed are more than the final dose given to patient because many of the solutions are *not completely infused*

Step	Solution	Rate (ml/hr)	Time (min)	Volume infused per step (ml)	Dose administered with this step (mg)	Cumulative dose (mg)
1	1	2.0	15	0.50	0.0160	0.0160
2	1	5.0	15	1.25	0.0400	0.0560
3	1	10.0	15	2.50	0.0800	0.1360
4	1	20.0	15	5.00	0.1600	0.2960
5	2	5.0	15	1.25	0.4000	0.6960
6	2	10.0	15	2.50	0.8000	1.4960
7	2	20.0	15	5.00	1.6000	3.0960
8	2	40.0	15	10.00	3.2000	6.2960
9	3	10.0	15	2.50	7.9370	14.2330
10	3	20.0	15	5.00	15.8741	30.1071
11	3	40.0	15	10.00	31.7482	61.8553
12	3	80.0	174.375	232.50	738.1447	800.0000

		Total time (minutes) =	339.375	= 5.66 hrs		

Figure 4. Evolution of severity of reactions during desensitization before and after acetylsalicylicacid (ASA)/montelukast pretreatment. Under the ASA and montelukast protocol, 86% of patients were able to tolerate further desensitizations, with a less severe hypersensitivity reaction or no reaction (grade 2.14 vs grade 0.5, *P* _ .001). Adapted from Breslow et al. [62]

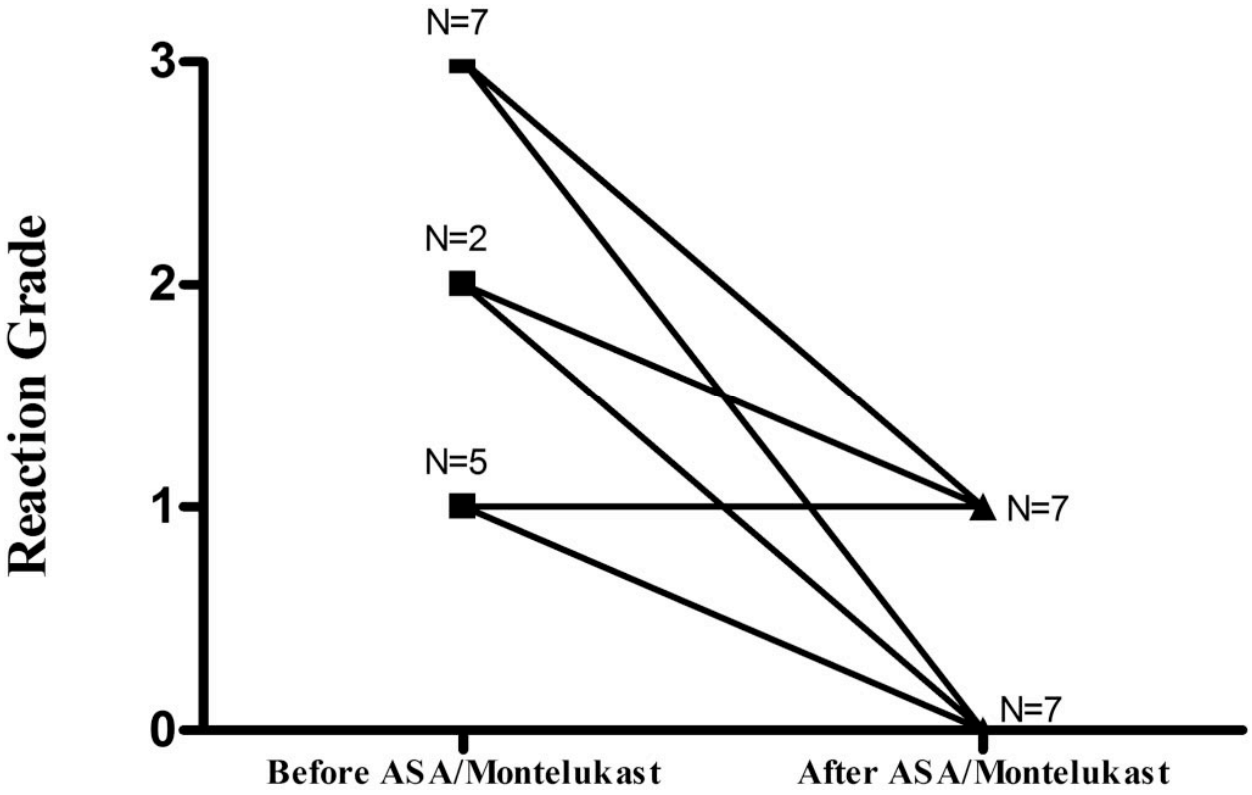
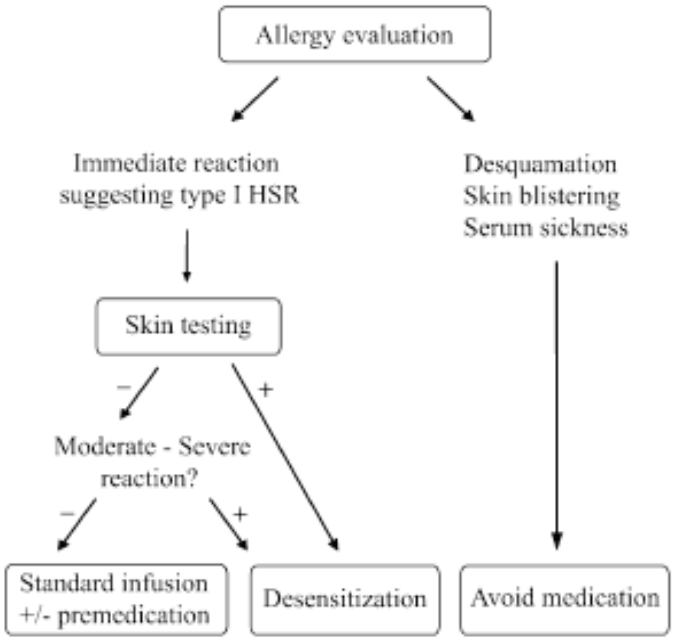


Figure 5. Algorithm for assessment and treatment of patients with hypersensitivity reactions to medications. Adapted from Brennan et al. [59]



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