

Antigen-IgE desensitization in signal transducer and activator of transcription 6–deficient mast cells by suboptimal doses of antigen

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Background: Rapid administration of suboptimal antigen induces transient unresponsiveness in patients with IgE antibodies to beneficial medications, but the molecular mechanisms of desensitization are poorly understood. Mast cells (MCs) have been implicated as the target cells.

Objective: To establish a physiologic model of IgE-antigen desensitization using mouse bone marrow–derived MCs (mBMMCs) from wild-type and signal transducer and activator of transcription 6 (STAT6)–deficient mice.

Methods: The mBMMCs were sensitized with dinitrophenyl (DNP) IgE or trinitrophenyl (TNP) IgE and activated with DNP/TNP–human serum albumin. For desensitization, suboptimal doses of DNP/TNP–human serum albumin were administered at fixed intervals.

Results: Desensitized mBMMCs failed to respond to an optimal dose of antigen, indicating successful desensitization. Desensitization was time dependent, with 5 minutes of antigen exposure being optimal. Resensitization with DNP-IgE did not reverse the process. The desensitized cells were responsive to calcium ionophore and phorbol myristate acetate. Thus, the desensitization reaction alters an early event in the high-affinity IgE receptor (FcεRI)–dependent signaling pathway in a nontoxic manner. The mBMMCs from STAT6-null mice could not be desensitized by suboptimal doses of antigen.

Conclusions: Mast cells can be rendered unresponsive by rapid administration of suboptimal doses of antigen in the presence of calcium, similar to in vivo desensitizations. The STAT6-null mBMMCs cannot be desensitized, providing the first molecular target in this inhibitory process.

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INTRODUCTION

Rapid desensitization provides temporary unresponsiveness to antigens, thereby allowing patients to be treated with medications to which they have had IgE-mediated adverse reactions.^{1–5} The rapid administration (minutes to hours) of suboptimal doses of antigen renders patients temporarily unresponsive to full therapeutic doses.⁶ Although desensitizations remain empirical and pose a high risk by reintroducing a potentially lethal medication in a short time into the allergic patient, the demand for such procedures has increased in the past 10 years.^{7–10}

Despite their clinical success, little is known concerning the mechanisms and molecular targets of antigen-IgE desensitization and its time requirements. A role for tissue mast cells (MCs) has been established in vivo because skin MCs become unreactive to the desensitizing medication once desensitization has been achieved.^{8,11,12} Although multiple successful rapid desensitization protocols have been reported,^{8,13} the reintroduction of progressively increasing suboptimal doses of allergen requires continuous or fixed intervals, resembling late-phase MC reactions. We therefore looked for

molecules implicated in early- and late-phase MC activation responses through the high-affinity IgE receptor (FcεRI). Signal transducer and activator of transcription 6 (STAT6)–deficient mouse bone marrow–derived MCs (mBMMCs) can be activated by antigen cross-linking of FcεRI receptors but cannot release late-phase cytokines. This indicates that MC late-phase responses that require transcription, translation, or molecular processing after FcεRI triggering are impaired.¹⁴ Importantly, STAT6 plays a central role in interleukin 4 (IL-4)–regulated responses in T cells,¹⁵ B cells,¹⁶ and MCs.¹⁷ The STAT6-null mice are viable but do not develop T_H2 responses,¹⁸ and B cells do not make IgE or express CD23 or major histocompatibility complex class II molecules.¹⁵ We established a model of rapid IgE-antigen desensitization in wild-type and STAT6-deficient mBMMCs. Administration of increasing or fixed amounts of suboptimal doses of antigen at fixed intervals induced unresponsiveness to optimal doses in wild-type mBMMCs. Using this model, we show that STAT6 plays a key role in the desensitization process.

METHODS

Monoclonal anti-dinitrophenyl (DNP) IgE clone SPE-7 (DNP-IgE), globulin-free human serum albumin (HSA), and DNP-HSA were from Sigma-Aldrich Corp (St Louis, MO). Recombinant mouse IL-3, monoclonal anti-trinitrophenyl (TNP) IgE clone TIB-142 (TNP-IgE), and TNP-HSA were a

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gift from Michael Gurish, PhD (Harvard Medical School). The β -hexosaminidase stop solution (0.2M glycine, pH 10.7) and the β -hexosaminidase cocktail (0.2M disodium hydrogen phosphate dodecahydrate; 0.4M citric acid water, pH 4.5; and 4-mmol/L p-nitrophenyl- β -D-2-acetamido-2-deoxy-glycopyranoside) were made.

Activation and Desensitization of mBMMCs

Mouse bone marrow–derived MCs were developed¹⁹ by culturing bone marrow progenitors from wild-type BALB/c and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) and from STAT6-null mice on both genetic backgrounds (from Michael Grusby, PhD, Harvard School of Public Health) in IL-3–enriched culture medium. Eight- to 12-week-old mBMMCs were sensitized with 0.25 μ g of anti-DNP–IgE (clone SPE-7)/10⁶ cells or anti-TNP–IgE (clone TIB-142) overnight at 37°C, washed twice by centrifugation at 4°C for 5 minutes at 1,000 rpm, and resuspended in fresh medium. Cells were placed in 5-mL polystyrene tubes in a 37°C bath at 50 μ L/10⁶ cells and challenged with 50 μ L of DNP-HSA, TNP-HSA, or HSA at 20 ng/mL (total dose, 1 ng) at 5, 50, or 100 minutes. Activation with calcium ionophore A23187 at 10 μ M and phorbol myristate acetate (PMA) at 10 ng/mL of DNP-IgE–sensitized mBMMCs was performed by adding 25 μ L of each for 5 minutes. β -Hexosaminidase release was evaluated after 5 minutes (see the following subsection).

For desensitization, mBMMCs were sensitized with DNP-IgE or TNP-IgE as described previously herein. Increasing concentrations of DNP-HSA or TNP-HSA (50 μ L) as follows: 1, 2, 4, and 8 μ L at 1 pg/ μ L; 0.5 (12 pg), 1 (32 pg), 2 (64 pg), 4 (128 pg), and 8 (256 pg) μ L at 32 pg/ μ L; and then 20 μ L at 25 μ g/mL (500 pg) or fixed (1 pg/1 μ L) concentrations followed by 1 ng were added by gentle addition of drops to each tube at 37°C at varying intervals. The mBMMCs were then processed for β -hexosaminidase release (see the following subsection). Cell viability at the end of desensitization was evaluated by trypan blue exclusion.

β -Hexosaminidase Release

After activation or desensitization, cells were centrifuged to separate supernatants and were resuspended in 100 μ L 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. Twenty microliters from each supernatant and pellet in duplicate were added to 80 μ L 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 μ L of β -hexosaminidase stop solution was added, and fluorescence was read at 405 nm on a kinetic microplate enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA). The net percentage of β -hexosaminidase release was $[S/(S+P)] \times 100$, where S and P are the β -hexosaminidase contents of each supernatant and cell pellet.

Statistical Analysis

Comparison between 2 experimental conditions was performed using the 2-tailed t test. Statistical significance was set at $P < .05$.

RESULTS

Rapid Desensitization of mBMMCs by Doubling Doses of Antigen Is Time Dependent

We used 2 different antigens for the evaluation of activation and desensitization of mBMMCs. The activation of DNP-IgE– or TNP-IgE–sensitized BMMCs with DNP-HSA or TNP-HSA induced a significant release of β -hexosaminidase compared with the activation with vehicle HSA alone (Fig 1). To control for the time of duration of desensitization and its impact in cell viability and activation capacity, we activated sensitized BMMCs after waiting 50 and 100 minutes at 37°C along with desensitized cells. There was not a statistically significant difference between those cells and cells activated for only 5 minutes (shown for TNP-IgE–sensitized BMMCs challenged with TNP-HSA after 100 minutes) (Fig 1). The administration of 10 doubling doses of DNP-HSA to DNP-IgE–sensitized cells, starting at 1 pg and delivering a total of more than 1 ng (1,011 pg), induced increasing unresponsiveness as the time between doses was increased from 1 to 5 minutes (Fig 1). There was no statistically significant difference between the release of β -hexosaminidase in cells activated for 5 minutes with DNP-HSA and desensitized cells when doses were added at 1-, 2-, 3- and 4-minute intervals. A statistically significant difference was seen between cells

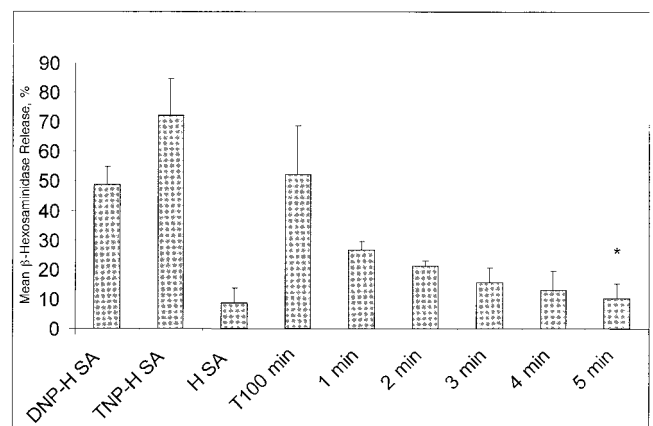


Figure 1. Rapid desensitization of mouse bone marrow–derived mast cells (mBMMCs) by doubling doses of dinitrophenyl (DNP)/trinitrophenyl (TNP) human serum albumin (HSA). BALB/c mBMMCs were sensitized with DNP-IgE or TNP-IgE overnight. In columns 1 to 3, DNP-HSA, TNP-HSA, or HSA (1 ng) was added to mBMMCs sensitized with DNP-IgE (columns 1, 3, and 5–9) or TNP-IgE (columns 2 and 4 [100 minutes]). Desensitization was accomplished by adding 10 doubling doses of DNP-HSA starting at 1 pg and with a total dose of 1 ng (columns 5–9). Doses were delivered at 1-, 2-, 3-, 4-, and 5-minute intervals (columns 5–9). β -Hexosaminidase release was measured in 3 experiments. The asterisk indicates statistical significance. Error bars represent SD.

desensitized with 10 doses of DNP-HSA at 5-minute intervals and cells activated for 5 minutes ($P = .02$). Cell viability at 50 minutes was greater than 95%. At 10-minute intervals, desensitization was not increased (data not shown); thus, 5-minute intervals were chosen as optimal for the remainder of the study.

Rapid Desensitization of mBMMCs by Repetitive

Suboptimal Doses of Antigen Is Dose and Time Dependent

Because desensitization of mBMMCs in calcium-depleted medium can be accomplished by a single suboptimal dose of DNP-HSA,²⁰ we hypothesized that a dose threshold requirement might exist for calcium-dependent desensitization. The administration of multiple doses of 1 pg, at 5-minute intervals, induced progressive unresponsiveness. Columns 3 through 11 in Figure 2 represent the release of β -hexosaminidase from cells receiving 2, 4, 6, 8, 10, 12, 14, 16, and 18 consecutive 1-pg doses, respectively, followed by a final dose of 1 ng. There was no statistically significant difference between the release of β -hexosaminidase from cells activated for 5 minutes with DNP-HSA and desensitized cells receiving 2, 4, 6, 8, 10, 12, and 14 doses (1 pg each) of DNP-HSA (Fig 2). There was a statistically significant difference between cells desensitized with 16 and 18 doses of DNP-HSA at 5-minute intervals and cells activated for 5 minutes ($P = .01$ for both) (Fig 2). Parallel experiments performed with TNP-IgE-sensitized BMMCs desensitized with TNP-HSA showed similar results (data not shown).

Rapidly Desensitized mBMMCs Remain Responsive to PMA and Calcium Ionophore

To evaluate whether rapid desensitization would deplete granule stores or would block the ability of mBMMCs to respond to other non-IgE-activating stimuli, mBMMCs were

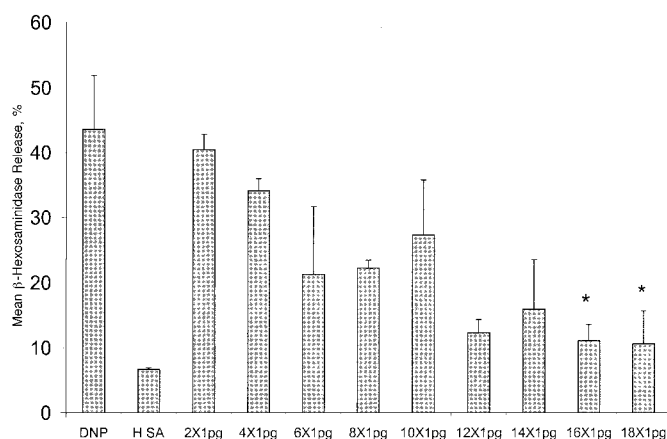


Figure 2. Rapid desensitization of mouse bone marrow-derived mast cells (mBMMCs) by single repetitive 1-pg doses of dinitrophenyl (DNP) human serum albumin (HSA). BALB/c mBMMCs were sensitized with DNP-IgE overnight. In columns 1 and 2, DNP-HSA or HSA (1 pg) was added to DNP-IgE-sensitized mBMMCs. In columns 3 to 11, 2 to 18 $\times$ 1 pg doses of DNP-HSA followed by a final dose of 1 ng were delivered at 5-minute intervals. β -Hexosaminidase release was measured in 3 experiments. The asterisk indicates statistical significance. Error bars represent SD.

stimulated by PMA and calcium ionophore A23187 after desensitization. In Figure 3, mBMMCs desensitized with 10 doubling doses of DNP-HSA at 5-minute intervals released statistically significantly less β -hexosaminidase than cells activated for 5 minutes. Similarly, desensitized cells treated with 10 μ M A23187 and 10 ng/mL of PMA for 15 minutes after desensitization released similar amounts of β -hexosaminidase as cells activated by 10 μ M A23187 and 10 ng/mL of PMA without desensitization (Fig 3). This indicated that the desensitization process neither depleted the β -hexosaminidase stores nor induced a toxic process preventing releases of mediators through a non-IgE stimulus.

Rapid Desensitization of mBMMCs Is Not Immediately Reversible by Resensitization With IgE

To determine whether resensitization would restore responsiveness immediately after rapid desensitization by providing new IgE/Fc ϵ RI receptor availability, we rapidly desensitized mBMMCs, resensitized the cells with DNP-IgE, and rechallenged with an optimal dose of DNP-HSA. As seen in Figure 4, resensitization with DNP-IgE of desensitized mBMMCs did not reverse desensitization, which was similar to that of nonresensitized desensitized cells. This indicated that receptor availability was not limiting once desensitization had been achieved.

The mBMMCs From STAT6-Null Mice Exhibit Dose-Dependent DNP-HSA Antigen Activation

We tested whether the early phase of the activation events was altered in STAT6-deficient BMMCs. As shown in Figure 5, there was a dose-dependent response of β -hexosaminidase re-

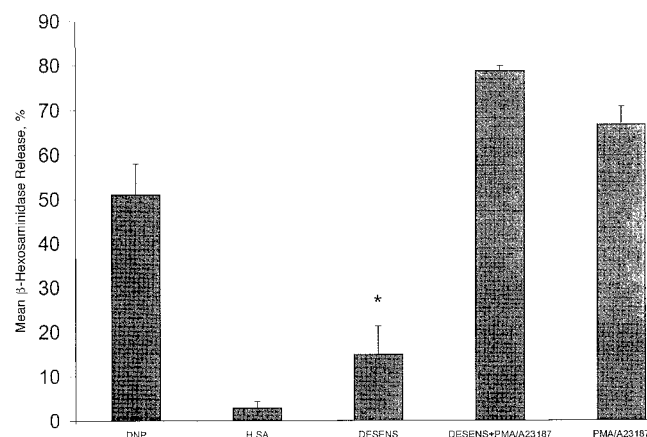


Figure 3. Rapidly desensitized mouse bone marrow-derived mast cells (mBMMCs) by increasing doubling doses of dinitrophenyl (DNP) human serum albumin (HSA) remained responsive to phorbol myristate acetate (PMA) and calcium ionophore. BALB/c mBMMCs were sensitized with DNP-IgE overnight and challenged with DNP-HSA or HSA (1 ng) (columns 1 and 2) or 10 μ M A23187 and 10 ng/mL of PMA (column 5). Cells were desensitized (DESENS) with doubling doses of DNP-HSA (columns 3 and 4) at 5-minute intervals and rechallenged with 10 μ M A23187 and 10 ng/mL of PMA (column 4). β -Hexosaminidase release was measured in 3 experiments. The asterisk indicates statistical significance. Error bars represent SD.

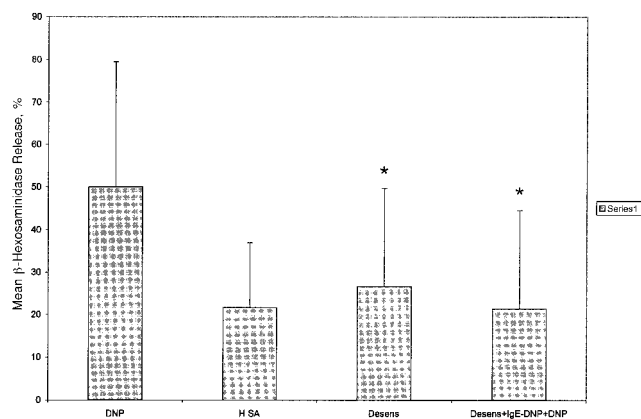


Figure 4. Rapid desensitization of mouse bone marrow–derived mast cells (mBMMCs) is not reversible by resensitization with dinitrophenyl (DNP) IgE. BALB/c mBMMCs were sensitized overnight with DNP-IgE and challenged with DNP human serum albumin (HSA) or HSA (1 ng) (columns 1 and 2). Sensitized cells were desensitized (DESENS) with 18×1 pg doses of DNP-HSA at 5-minute intervals, followed by 1 ng (columns 3 and 4). Resensitization with $0.25 \mu\text{g}$ of DNP-IgE for 30 minutes and challenge with 1 ng of DNP-HSA was performed in column 4. β -Hexosaminidase release was measured in 3 experiments. The asterisk indicates statistical significance. Error bars represent SD.

lease to increasing single doses of DNP-HSA in STAT6-deficient mBMMCs, comparable with the response of mBMMCs from wild-type mice (data not shown), indicating that the early response to IgE and antigen was preserved and was not enhanced.

The mBMMCs From STAT6-Null Mice Cannot Be Rapidly Desensitized by Suboptimal Doses of Antigen

We evaluated the capacity for rapid desensitization of mBMMCs from two genetic backgrounds, BALB/c and

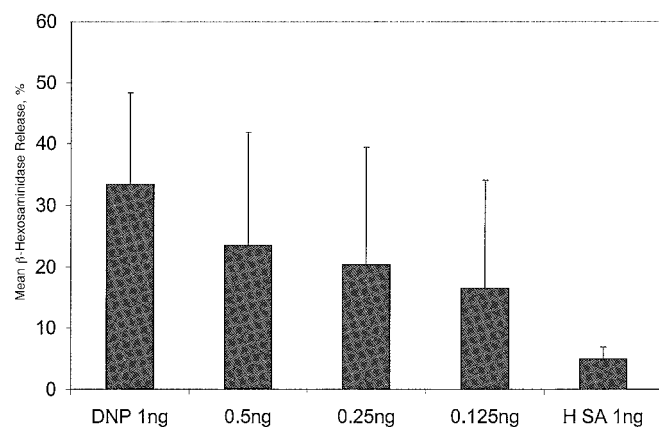


Figure 5. Mouse bone marrow–derived mast cells (mBMMCs) from signal transducer and activator of transcription 6 (STAT6) BALB/c-null mice exhibit dose-dependent activation with dinitrophenyl (DNP) human serum albumin (HSA) comparable with that of wild-type mBMMCs. BALB/c STAT6-null mBMMCs were sensitized overnight with DNP-IgE. Challenge was performed with DNP-HSA at 1, 0.5, 0.25, and 0.125 ng (columns 1–4) or HSA at 1 ng (column 5). β -Hexosaminidase release was measured in 3 experiments. Error bars represent SD.

C57BL/6, from STAT6-null mice compared with wild-type mBMMCs. As shown in Figure 6, after desensitization, the release of β -hexosaminidase mBMMCs from BALB/c STAT6-null mice and mBMMCs from C57BL/6 STAT6-null mice had no statistically significant difference with activated cells, indicating a failure of desensitization. In contrast, as seen in Figure 6B, similarly treated mBMMCs from wild-type C57BL/6 mice were successfully desensitized by repetitive 1-pg doses of DNP-HSA at 5-minute intervals, indicating that STAT6 provided an essential mechanism for desensitization.

DISCUSSION

The present study describes a physiologic model of rapid MC desensitization to antigen in the presence of calcium that can be applicable to the study of human desensitizations. This model provides insight into the time requirements for desensitization and describes its first molecular target, the transcription factor STAT6.

The initial 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.^{1,5} More recent protocols¹³ have provided support for these early empirical starting doses and for the need for interval times between doses. Based on

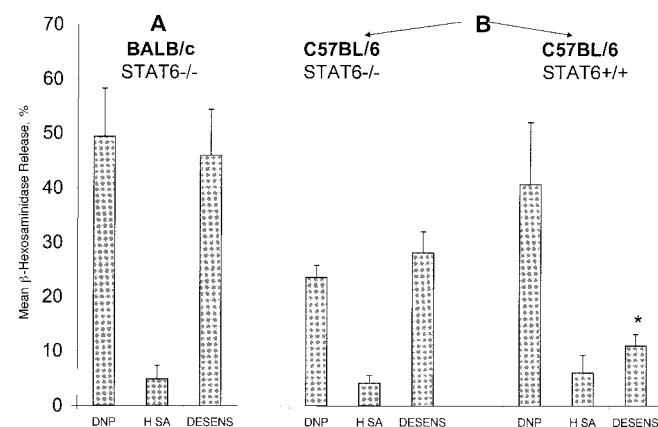


Figure 6. Signal transducer and activator of transcription 6 (STAT6)–null mouse bone marrow–derived mast cells (mBMMCs) from BALB/c and C57BL/6 mice cannot be rapidly desensitized to dinitrophenyl (DNP) human serum albumin (HSA). BALB/c and C57BL/6 mBMMCs from STAT6-null mice and C57BL/6 mBMMCs from wild-type mice were sensitized overnight with DNP-IgE and challenged with 1 ng of DNP-HSA or HSA and desensitized with 18×1 pg doses followed by 1 ng. A, The mBMMCs from BALB/c STAT6-null mice were activated with DNP-HSA (column 1), HSA (column 2), or desensitized (DESENS) (column 3). B, On the left, mBMMCs from C57BL/6 STAT6-null mice were activated with DNP-HSA (column 4), HSA (column 5), or desensitized (column 6); on the right, mBMMCs from wild-type C57BL/6 mice were activated with DNP-HSA (column 7), HSA (column 8), or desensitized (column 9). β -Hexosaminidase release was measured in 3 experiments. The asterisk indicates statistical significance. Error bars represent SD.

the human data, we proceeded to generate an in vitro protocol of desensitization using mBMMCs and an initial dose of 1/1,000 the target dose (1 pg) followed by suboptimal doses of DNP-HSA until reaching the target dose of 1 ng. Unresponsiveness to the optimal antigen dose of 1 ng was achieved with 10 doubling doses and was time dependent because progression from 1- to 5-minute intervals between doses yielded progressively lower β -hexosaminidase release. Statistically significant differences were seen between activated and desensitized cells when 5 minutes was used, with a total of 50 minutes for the desensitization process. Delivering doses too fast delayed unresponsiveness, with substantial release of β -hexosaminidase when antigen was delivered at 1-minute (26.7%), 2-minute (21.2%), 3-minute (15.6%), and 4-minute (13.1%) intervals (Fig 1). (This could be the reflection of breakthrough reactions during human desensitizations, when the delivery of suboptimal doses of antigen starts at a dose that is too high or antigen doses are delivered at short intervals.) In our model, when doses were delivered at 5-minute intervals, the release was 10.18%, not statistically different from the release with HSA (8.7%).

A threshold dose of suboptimal antigen is critical during desensitizations, as shown in vitro in calcium-dependent desensitization^{21,22} of human foreskin MCs and in calcium-independent desensitization of mBMMCs²⁰ and human basophils.^{23–25} We identified a threshold in our model by using a single repetitive dose challenge of 1 pg until reaching complete unresponsiveness. In this system, 16 and 18 $\times$ 1 pg single repetitive doses triggered significant unresponsiveness to an optimal antigen challenge of 1 ng (Fig 2). After 16 and 18 pg, the cells became unresponsive to 1 ng, indicating that the necessary threshold of antigen had been reached. This suggests that desensitization is not a linear process and that the addition of further antigen, once the threshold is reached, is not necessary because unresponsiveness is already achieved. A study²² of rat peritoneal MCs co-cultured with 3T3 fibroblasts indicated that 10-fold increasing doses of DNP-HSA administered at 30-minute intervals render the cells unresponsive to 100 ng. Similarly, desensitization of sensitized human basophils performed by incubation with 1 dose of anti-IgE or Rye 1 antigen for up to 30 minutes triggered unresponsiveness to optimal doses.^{26,27} During rapid MC desensitization, a minimal threshold of antigen needs to be delivered before cells become unresponsive, and the threshold can be as low as 1/50 the target dose. A mathematical analysis of calcium-independent desensitization of passively sensitized human basophils with serum from penicillin-allergic patients indicated that longer incubation times (30 minutes) with suboptimal doses of penicillin induced maximal unresponsiveness (<10% release of histamine) compared with shorter times (15 minutes) (20% release). Furthermore, the data support the notion that a threshold dose of antigen cross-linking is required for desensitization,²⁸ as indicated by the present studies. Basophils isolated from 1 patient desensitized to penicillin who had a negative skin test result were responsive to penicillin in vitro.¹² Although this finding sug-

gests that IgE-bearing desensitized basophils lose their inhibitory capacity when exposed to antigen in vitro, a more likely explanation is that basophils do not represent tissue MCs.

Desensitized mBMMCs responded to PMA and the calcium ionophore A23187 (Fig 3), indicating that no depletion of mediators had occurred and that the pathway of non-IgE-mediated activation was intact. Similarly, DNP-HSA-desensitized rat peritoneal MCs co-cultured with 3T3 fibroblasts remained responsive to calcium secretagogue 48/80.²² Desensitized mBMMCs remained unresponsive after resensitization (Fig 4) to a second challenge with the optimal dose of antigen, indicating that the defect in responsiveness was not likely due to lack of receptor occupancy. This is consistent with a study²⁷ of RBL-3T3 cells in which IgE-DNP-sensitized cells were rendered unresponsive to further cross-linking with DNP-bovine serum albumin by desensitization.

Because of the requirement for a critical amount of time between doses to achieve successful rapid desensitization, we turn our attention to molecules involved in the late phase of the IgE-antigen response, in particular to STAT6. The STAT6-deficient mBMMCs, which can be activated by antigen cross-linking of Fc ϵ RI receptors, cannot release late-phase cytokines.¹⁴ The STAT6-null mice are viable but do not develop T_H2 responses,¹⁸ and B cells do not make IgE or express CD23 or major histocompatibility complex class II molecules.¹⁵ Interleukin 4 controls the expression of Fc ϵ RI in some populations of cultured MCs via a STAT6-dependent mechanism.¹⁷ However, the fact that STAT6-null mBMMCs develop normally and express normal numbers of Fc ϵ RI receptors suggests that alternative pathways may provide control for the in vivo expression of the high-affinity IgE receptor.¹⁴ Mast cells are responsive to IL-4, and the IL-4 receptor transduces some of its signals via a proteolytic fragment of STAT6. We attempted desensitization of STAT6 mBMMCs from 2 genetic backgrounds, and, to our surprise, STAT6-null mBMMCs could not be desensitized by either doubling doses or repetitive suboptimal doses of DNP-HSA (Fig 6), indicating that STAT6 is necessary during rapid desensitization. Early activation of these cells is comparable to wild-type mBMMCs, as seen in Figure 5, indicating that signal transduction during the early phase of Fc ϵ RI activation remains intact. Whether STAT6 has a direct role or provides a regulatory inhibitory factor(s) during desensitization needs to be further explored. In MCs, the IL-4 receptor transduces some of its signals via a proteolytic 65-kDa fragment of STAT6,^{29,30} cleaved by an undefined nucleus-associated protease. Early data on calcium-independent desensitization of peritoneal MCs had implicated a serine protease during desensitization to antigen.²⁰ We speculate that STAT6 may be the substrate of the undefined serine protease and that the production of the short 65-kDa STAT6 form may be the rate-limiting factor of desensitization.

For patients who experience IgE-mediated life-threatening reactions to β -lactams and other beneficial medications, it is essential to understand at the molecular level the mechanisms that lead to antigen desensitization. Our studies provide an in

vitro model of physiologic MC desensitization that can be used for the study of human desensitizations. We also present dose and time parameters of successful rapid desensitizations. We show that a low threshold of antigen is necessary before unresponsiveness is reached, which can be as low as 1/50 the target dose. We provide evidence that a critical amount of time is needed between the administration of antigen doses to provide total unresponsiveness to optimal doses of target antigen and that STAT6 is directly or indirectly implicated in the inhibitory process.

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