Immunologic characterization of 3 murine regimens of allergen-specific immunotherapy

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Background: Allergen-specific immunotherapy (ASIT) is used to treat the symptoms of immediate type I hypersensitivity. The mechanisms driving establishment of allergen tolerance are not yet fully understood.
Objective: The goal of this study was to develop and immunologically characterize 3 murine models of ASIT to simulate protocols currently used to treat patients with type I hypersensitivities.
Methods: Ovalbumin (OVA)–sensitized mice were desensitized to OVA by means of repeated injections of OVA with a rapid, intermediate, or gradual protocol. After desensitization, mice were assessed for clinical sensitivity to OVA, and immunologic parameters were assessed.
Results: Mice in all treatment protocols displayed decreased vascular permeability in response to OVA challenge after desensitization. Circulating OVA-specific IgE levels, as well as basophil activation in response to OVA stimulation and IgE cross-linking, were significantly decreased in all treatment groups. Intermediate and gradual protocols, but not rapid desensitization, suppressed splenocyte proliferation and production of IL-4, IL-5, and IFN-γ in response to OVA and polyclonal activation. Similarly, significant increases in IL-10 production, numbers of $CD4^+CD25^+$ forkhead box protein 3–positive regulatory T cells, and OVA-specific IgG_1 antibody levels were only observed in mice undergoing prolonged ASIT regimens.
Conclusion: Suppression of IgE-mediated activation is a common feature of all desensitization schedules. Induction of immunoregulatory networks requires prolonged desensitization schedules. (J Allergy Clin Immunol 2015;135:1341-51.)

Key words: *Type I hypersensitivity, allergen-specific immunotherapy, mouse models, mast cells, basophils*

Abbreviations used
ASIT: Allergen-specific immunotherapy
BrdU: 5-Bromo-2′-deoxyuridine
FoxP3: Forkhead box protein 3
OVA: Ovalbumin
PerCP: Peridinin-chlorophyll-protein complex
Treg: Regulatory T

Allergen-specific immunotherapy (ASIT) is a highly effective antigen-specific treatment for IgE-mediated allergic diseases, such as allergic rhinitis, drug sensitivity, and venom hypersensitivity.[1] Unlike pharmacologic treatments available for symptoms of allergies (eg, antihistamines), ASIT prevents the development of severe allergic responses and, when administered for 2 to 5 years, can provide long-lasting protection.[2,3]

Clinically, ASIT achieves tolerance through the use of repeated administration of allergens. Although specific protocols vary from clinician to clinician, there are 3 types of ASIT commonly used in the United States. Conventional immunotherapy, which is most often used to treat seasonal allergic rhinitis, involves 1 to 2 injections a week until the maintenance dose is reached.[4] Alternatively, patients might undergo cluster or rush immunotherapy, which is commonly used to treat venom and drug hypersensitivity. Cluster immunotherapy schedules involve several injections a day on nonconsecutive days until the maintenance dose is reached in approximately 4 to 8 weeks, whereas rush schedules involve injections of antigen every 15 to 60 minutes over a period of 1 to 3 days to achieve the maintenance dose.[5]

Although there are a number of mechanisms that might play a role in diminished clinical responses after ASIT, no single hypothesis is completely sufficient to explain diminished allergic reactivity to antigen challenge. One theory postulates that a switch from a T_H2 phenotype to a T_H1 phenotype is a key mechanism in ASIT, but observations during clinical studies have not definitively demonstrated phenotype switching to be the principal mechanism.[6-11] Additionally, changes in circulating IgE and IgG levels have been observed after successful ASIT but do not always correlate with achievement of clinical benefit.[12] Studies in patient populations have demonstrated that regulatory T (Treg) cells might play a role in establishing tolerance to allergens, but, similar to antibody responses, the development of an antigen-specific Treg cell response does not always correlate with the clinical benefit after ASIT, especially in accelerated immunotherapy schedules.[13]

The goal of this study was to develop and characterize 3 different murine models of ASIT to simulate protocols currently used to treat patients for allergen hypersensitivities. These models provide insights into the biology of ASIT and can function as tools to further elucidate the mechanisms involved in establishing immune tolerance to allergens.

METHODS

Animals

Female BALB/c (NCI Mouse Repository, Frederick, Md), $Wsh^{-/-}$ (The Jackson Laboratory, Bar Harbor, Me), and C57BL/6 (The Jackson Laboratory) mice were maintained at Uniformed Services University under a protocol

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approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee. At study termination, mice were killed with carbon dioxide, followed by cervical dislocation. Blood was collected by means of cardiac puncture.

Sensitization and desensitization of mice with ovalbumin

BALB/c mice were sensitized through administration of 50 μg of ovalbumin (OVA; Sigma, St Louis, Mo) adsorbed to Imject alum (Pierce, Rockford, Ill) by means of intraperitoneal injection every week for 3 weeks (total of 3 doses). After a 2-week resting period, mice were desensitized by means of intraperitoneal injection of one of 3 protocols with 100 μl/mL of a 500 μg/mL solution of OVA prepared in PBS for a final dose of 50 μg. Rapid desensitization consisted of 3 intraperitoneal OVA injections per day for 7 days (total of 21 doses). Intermediate desensitization consisted of intraperitoneal OVA injections every other day for 4 weeks (total of 14 doses). Gradual desensitization consisted of 1 OVA injection per week for 9 weeks (total of 9 doses). The protocols are outlined in Fig E1 (see this article's Online Repository at www.jacionline.org).

We chose these schedules to approximate desensitization protocols that are used clinically. Our rapid desensitization protocol was chosen to represent a rush/ultrarush desensitization protocol. To limit the number of intraperitoneal injections received by mice, we used 3 injections a day for 7 days. The gradual desensitization protocol was developed to simulate subjects undergoing conventional immunotherapy, a practice that generally consists of allergen doses administered 1 to 2 days a week for a period of weeks until the maintenance phase is reached. The intermediate desensitization protocol was developed as an intermediary model between the rapid and gradual desensitization. It approximates a clinical cluster protocol in which patients are given several injections a week on nonconsecutive days.

Additionally, one experiment used an escalating dosage regimen with the same frequency of injections as the rapid desensitization protocol. Termed "escalating rapid desensitization," this regimen started with 3 intraperitoneal injections of 0.78 μg of OVA on day 1 and then doubled the amount of OVA given per injection every day until a dosage of 50 μg was reached on the seventh and final day of treatment. The study end point (ie, the time point when clinical and immunologic studies were conducted) was 2 hours after the final OVA injection for all protocols.

Antihistamine administration

Fexofenadine HCl, an HR1 antagonist, was dissolved in drinking water at a concentration of 0.25 mg/mL for an average dosage of 20 mg/kg/d. Cimetidine (Sigma-Aldrich), an HR2 antagonist, was prepared by dissolving in HCl and mixed with water. The pH was then adjusted to 7.0 with NaOH. The final concentration of cimetidine was 2.5 mg/mL in drinking water, for an average dosage of 200 mg/kg/d. Drinking water bottles containing a 1:1 mix of fexofenadine and cimetidine were changed every other day. Antihistamine activity was confirmed by testing stomach pH at the time of death (for fexofenadine) and based on local anaphylaxis in response to a direct histamine challenge (for cimetidine).

Basophil depletion

In vivo depletion of basophils was performed, as previously described.[14] In brief, 50 μg of Ba103, a rat IgG_{2b} mAb that recognizes CD200R3, was injected intraperitoneally 3 days before OVA challenge.[15] Control mice were given injections of IgG_{2b} isotype control (BD Biosciences San Jose, Calif). Depletion of basophils was confirmed by means of flow cytometry.

Assessment of sensitivity based on local anaphylaxis

Sensitivity to OVA was determined based on local anaphylaxis, as previously described.[16] In short, mice were anesthetized with a mixture of 46 mg/kg body weight ketamine (Ketaject; Phoenix Pharmaceutical, St Joseph, Mo), 0.225 mg/kg acepromazine (PromAce Injectable; Fort Dodge Animal Health, Fort Dodge, Iowa), and 5 mg/kg xylazine (TranquiVed; Vedco, St Joseph, Mo), as previously described.[17] Ten microliters of a 500 μg/mL solution of OVA was injected intradermally into the ear. After 3 minutes, 200 μL of a 0.5% Evans Blue Dye solution was injected into the tail vein. Ten minutes later, mice were euthanized with CO_2 and cervical dislocation. Ears were dissected, and the dye was extracted from ears overnight in 700 μL of formamide at 60°C. Absorbance was read at 620 nm.

OVA-specific IgE, IgG_1, IgG_{2a}, and total IgE ELISA

Blood was collected in heparinized microcentrifuge tubes (Starstedt, Nümbrecht, Germany) from mice treated with OVA at various time points, and plasma was analyzed for levels of total IgE and OVA-specific IgE, IgG_1, and IgG_{2a}. For OVA-specific ELISA, flat-bottom Corning costar EIA plates (Corning Laboratories, Corning, NY) were coated overnight at 4°C with 20 μg/mL OVA. Nonspecific binding was blocked with 5% BSA/PBS and 0.05% Tween. For OVA-specific IgE ELISA, IgG was first depleted out of plasma by incubating serum samples with GammaBind G sepharose (Amersham Biosciences, Piscataway, NJ) overnight at 4°C. Samples were then centrifuged, and supernatants were used for immunoassay. Plasma samples were diluted 8 times by means of 5-fold serial dilutions in 1% BSA/PBS and plated in duplicate. Plates were then washed, and plate-bound IgE was detected by addition of biotinylated rat anti-mouse IgE (BD Biosciences) diluted in 1% BSA/PBS, followed by addition of alkaline phosphatase–conjugated streptavidin (dilution 1:1000) in 1% BSA/PBS. Plates were then washed, and 4-nitrophenyl phosphate disodium (Sigma-Aldrich) in 0.1 mol/L carbonate buffer was added to each well. Colorimetric development was detected at 405 nm by using a Victor V Microplate Reader (PerkinElmer, Waltham, Mass). Detection of total IgE was performed with identical steps as for OVA-specific IgE, except that plates were initially coated with 10 μg/mL anti-IgE (BD Biosciences) in PBS, and plasma samples were plated at dilutions of 1:10 and 1:90. Total IgE concentrations were determined by using an IgE standard curve (BD Biosciences) and WorkOut 2.0 ELISA software (PerkinElmer).

Detection of OVA-specific IgG_1 and IgG_{2a} was performed with identical steps as for OVA-specific IgE, except that plates were coated with 10 μg/mL anti-IgG_1 or IgG_{2a} (BD Biosciences), and IgG was not adsorbed out of plasma samples. Samples were diluted by means of 5-fold serial dilution and plated in duplicate. Plate-bound antibody was detected by addition of biotinylated anti-IgG_1 or IgG_{2a} (1:1000 dilution), followed by alkaline phosphate–conjugated streptavidin. Titers were defined as the greatest dilution that conferred an OD reading more than 1.5 times that of the PBS control.

Splenocyte stimulation and assessment of cytokine production

Single-cell suspensions of splenocytes were prepared by homogenizing spleens with the plunger of a 1-mL syringe and passing cells through a 70-μm cell strainer. Red blood cell lysis was achieved by treating the cell suspension with ACK lysis buffer (Invitrogen, Carlsbad, Calif), followed by washing. Cells were cultured at a concentration of 2×10^6 cells in 1 mL of IgE media (Iscove modified Dulbecco medium [Mediatech, Manassas, Va] with 10% FCS [Valley Biomedical, Winchester, Va], 1% L-glutamine [Mediatech], 1% insulin-transferrin-selenium medium [Invitrogen], and 80 μg/mL gentamicin [Invitrogen]). Cells were stimulated with media, OVA (20 μg/mL), or 5 μg/mL plate-bound α-CD3 (eBioscience, San Diego, Calif) and 2 μg/mL α-CD28 (eBioscience) at 37°C with 5% CO_2 for 72 hours. Culture supernatants were collected and kept at −20°C until ELISA could be performed. IL-4, IL-5, IFN-γ, and IL-10 levels were quantified by means of ELISA, according to the manufacturer's instructions (BD OptEIA, BD Biosciences).

Flow cytometric analysis of Treg cells

Cells were prepared for analysis, as previously described.[17] In brief, single-cell suspensions of spleens from mice were prepared as described above and brought to a final concentration of 1.0×10^7 cells in 5 mL of IgE media. Cells were cryopreserved in PBS/1% dimethyl sulfoxide (Sigma) until flow cytometric analysis could be performed.