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Innate basophil IL-4 responses against allergens, endotoxin, and cytokines require the Fc receptor γ-chain

To the Editor:

Basophils are regarded as important effector cells that contribute to allergic inflammation and induction of Th2 responses because they generate large amounts of IL-4 in IgE-dependent and IgE-independent manners.1-3 Although the IgE-dependent high-affinity IgE receptor (FcεRI)–mediated mechanisms of basophil and mast cell activation, in which the Fc receptor γ-chain (FcRγ) and spleen tyrosine kinase (Syk) play critical roles in intracellular signaling, have been examined extensively and characterized,4 information on the molecular mechanisms responsible for basophil IL-4 responses with various innate stimuli is very limited. Herein we demonstrate basophil IL-4 responses with an innate stimulation using allergenic pollen grains. Furthermore, we found that the basophil IL-4 responses induced by IgE-independent stimuli with allergen proteases, pollen, endotoxin, and cytokines all required FcRγ.

Bone marrow–derived (BM) basophils were prepared by culturing bone marrow cells from naive mice in the presence of IL-3 and purified (for further information, see the Methods section in this article’s Online Repository at www.jacionline.org). As demonstrated in our previous study,4 BM basophils from wild-type mice released IL-4 when stimulated with papain (Fig 1, A, left), a recombinant house dust mite (HDM) protease allergen (rDer f 1; Fig 1, B, left), or the IgE crosslink. IL-4 was also released by basophils stimulated with allergenic pollen grains (Fig 1, C, left). Because the signaling pathway for protease- or pollen-induced IL-4 release might differ from that for IgE crosslink–induced IL-4 release, which needs to be mediated by FcRγ, we examined IL-4 responses in BM basophils from FcRγ−/− mice. FcRγ−/− basophils did not release IL-4 when stimulated by the allergen proteases, pollen grains, or IgE crosslink (Fig 1, A-C, right panels). A stimulation in the presence of IL-3 increased IL-4 release by wild-type basophils but not by FcRγ−/− basophils (see Fig E1 in this article’s Online Repository at www.jacionline.org).
METHODS

Materials
Regado Biosciences (Basking Ridge, NJ) provided pegnivacogin and its component moieties, the 31 nucleotide un-PEGylated RNA aptamer (RB005, as noted in Fig 1, B) and a hexylamino 40-kDa branched mPEG used to produce pegnivacogin (mPEG2-NHS, Nektar, San Francisco, Calif), referred to in Fig 1 as HEXPEG. Pegloticase (PEGylated recombinant porcine urate oxidase) was obtained from Savient Pharmaceuticals (Bridgegewater, NJ). The 10-kDa mPEG-NPc (10 kDa p-nitrophenylcarbonate-activated monomethoxyPEG), the activated mPEG reagent used in the manufacture of pegloticase, was obtained from NOF (Tokyo, Japan). Pegylated bovine adenosine deaminase (Adagen) was obtained from Enzon Pharmaceuticals (Piscataway, NJ). Bovine spleen adenosine deaminase and 10-kDa PEG-diol were obtained from Sigma (St Louis, Mo).

Analytical methods

Testing for anti-PEG antibody. A protocol for studying anti-PEG antibodies in stored, coded, and deidentified samples from RADAR participants was granted an exemption from review by the Duke University Institutional Review Board on the basis that the proposed studies met “the definition of research not involving human subjects as described in 45 CFR 46.102(f), 21 CFR 56.102(e) and 21 CFR 812.3(p) and satisfies the Privacy Rule as described in 45CFR164.514.” The studies described in the present publication were initiated in December 2010 and completed in May 2011, although additional studies of some stored samples were performed more recently in preparing this report.

Initial screen of RADAR samples for anti-PEG antibody. An initial set of 98 deidentified and coded serum and plasma samples from 31 RADAR patients was screened at 1:21 dilution in 2 ELISAs, one to detect IgG binding to pegloticase and the second to detect binding to mPEG, using the manual procedure described previously. However, in the second ELISA, HEXPEG, the 40-kDa branched mPEG reagent used in producing pegnivacogin, replaced the linear 10-kDa mPEG-NPc reagent used in the manufacture of pegloticase. Positive samples were retested for confirmation, and specificity for PEG was further tested by direct and competition ELISAs to assess antibody binding to 10-kDa mPEG-NPc, pegnivacogin, RB005 (un-PEGylated aptamer), and PEGylated and un-PEGylated bovine adenosine deaminase. These assays were performed essentially as previously described.

In the competition ELISA, duplicate aliquots of samples (at final dilution 1:21) were preincubated with 0 to 20 μg of a competing antigen. The mixture was then carried through the antipegloticase ELISA. A significant decrease in A405 (compared with no-competitor control) indicated specificity for the competing antigen.

Reference standard and positive quality control (QC) used in ELISAs to detect anti-PEG antibodies were prepared from immune plasma of different individuals who had developed IgG antibodies with specificity toward PEG after receiving pegloticase in a phase 1 clinical trial. Negative QC consisted of pooled nonimmune plasma from 3 individuals in the same trial. Fig E1 illustrates the ability of these positive QC and reference standards to recognize molecular species relevant to the RADAR trial.

Automated testing for anti-PEG antibodies by competition ELISA. Following the initial 98-sample screen, further testing for anti-PEG antibody was carried out by the automated competition ELISA previously described, in which pegloticase was the plate coating and 20 μg of 10-kDa PEG-diol was the competitor. The cutoff points for the automated procedure were established by a statistical analysis of results obtained with 51 unique samples chosen randomly by the sponsor from among pretreatment samples from 328 patients who were not tested in the initial manual ELISA screen. From this analysis, the cutoff points established for the automated antipegloticase ELISA and competition steps were A405 values of 0.15% or more and 32% or more inhibition, respectively.

Anti-PEG antibody titer was estimated by end-point dilution in the antipegloticase ELISA by analyzing duplicate serial 2-fold dilutions from 1:10 to 1:2560. IgM and IgG isotypes were determined using μ- and γ-chain–specific secondary antibodies conjugated with alkaline phosphatase, and IgG subclass was determined with biotinylated mouse antihuman IgG1, IgG2, IgG3, and IgG4–specific antibodies (all from Sigma). Biotinylated antibodies were detected with the Vectastain ABC Kit AK-5000 (Vector Laboratories, Burlingame, Calif).

REG1-CLIN211a. REG1-CLIN211a was conducted as a follow-up to REGI-CLIN211 in the 3 subjects who experienced severe, acute allergic reactions following the administration of pegnivacogin. Of the potential participants, subjects 406-003 and 418-008 agreed to participate in this study, while the third subject, 602-004, was not contacted because of refusal of the investigative site to participate. Following approval of the protocol by local ethics committee and Regado, both subjects completed informed consent and had all assessments conducted during a single visit. The visit for each subject occurred on June 28, 2011, approximately 7 months and 8 months following dosing in the RADAR study. The following assessments were included in this study: questionnaires for allergy history, family history, complete medical history and exposure (environmental and ingestion) history, chemistry, hematology and urinalysis panels, hepatitis screening, anti-PEG antibody determination, and HLA typing.

No findings common to both were identified with respect to exposure, or specific allergy or medical history, except that both had experienced drug allergies with respiratory and cutaneous manifestations before the RADAR trial. Unfortunately, the frequency of drug allergies among other RADAR trial participants is not known. Both subjects remained positive for anti-PEG antibodies at follow-up, although antibody levels were lower than at dosing with pegnivacogin. Thus, when analyzed simultaneously by the automated method, A405 for pretreatment versus posttreatment samples were, respectively, 0.98 versus 0.37 for patient 406-003 and 1.83 versus 0.30 for patient 418-008.

Clinical database investigation. All terms defined as anaphylaxis by the National Institute of Allergy and Infectious Diseases and by the American Academy of Allergy, Asthma & Immunology guidance on drug allergy practice parameters were searched within the RADAR database from each individual clinical study location. All terms that met these criteria were then further investigated and included if there was a temporal relationship to REGI (either component) administration, if the signs or symptoms matched those expected for a “hypersensitivity” event, and if they were consistent with the pharmacokinetic properties of pegnivacogin or anivamersen. The occurrence of the 3 known allergic-like SAEs within 5 to 25 minutes after pegnivacogin administration, coupled with the fact that maximal plasma concentrations of pegnivacogin occur within 1 to 10 minutes after intravenous (IV) bolus, led us to conclude that relevant anaphylactoid reactions will occur within the first hour after pegnivacogin dosing. Based on these criteria, a cutoff of 2 hours from administration was used to define and further delineate allergic events “possibly related” to pegnivacogin. Beside the 3 SARs identified during the trial, review of the RADAR clinical data and data from previous phase 1 studies (n = 169) did not reveal a pattern of hypersensitivity events associated with pegnivacogin. Thus, the estimated incidence rate for serious allergic-like reactions to pegnivacogin in RADAR and earlier clinical trials was 0.6% (n = 3), with 0.4% (n = 2) meeting criteria for anaphylaxis.

Chemistry, Manufacturing and Controls investigation of pegnivacogin. The samples tested consisted of retention samples from each point along the study supply chain, including the active pharmaceutical ingredient (API) manufacturing site, the drug product (DP) fill/finish site, DP labeling and distribution centers (the United States and the European Union), and unopened clinical vials from sites at which the serious allergic-like reactions occurred. Additional control samples analyzed for comparative purposes included an API lot used in previous toxicology studies and 5 expired lots of pegnivacogin clinical trials material (CTMs).

Release testing was performed on all samples. Release tests included AX-HPLC to quantify purity and impurities, GPC-HPLC to determine molecular weight, assay to determine product strength, and endotoxin, pH, and osmolality determination per USP. Ongoing stability studies of both lots
of CTMs used in the RADAR trial provided sterility and particulate per USP, as well as appearance and activity test results from pull times that bracketed the SARs. Additional testing on DP retains and selected control samples included nuclear magnetic resonance (NMR) to address potential degradation or contamination that might not be observed by the release methods, and headspace GCMS to screen for low molecular weight impurities that may not be observed by NMR. The potential for pegnivacogin to aggregate was assessed by NMR and GPC on API and DP manufacturing retains. All tests were conducted under protocol. All CTMs analyzed met specifications, and the additional nonrelease testing did not identify any unexpected impurities or evidence of product aggregation.

Safety pharmacology study in cynomolgus monkeys. This was a GLP study conducted at MPI (Mattawan, Mich) per its SOPs with inspections conducted by the MPI Quality Assurance Department.

Animals (n = 6) were surgically instrumented with venous vascular access ports for dosing and blood collection and were also surgically prepared with telemetry transmitters for measurement of respiratory and cardiovascular parameters. One group of 6 monkeys was sequentially administered a single IV dose of the vehicle (PBS, pH 7.4) on day 1, followed by a single IV dose of the intact pegnivacogin (20 mg/kg) on day 3, and then a single IV dose of the expired pegnivacogin (20 mg/kg) on day 15. All doses were administered via the vascular access ports at a dose volume of 0.95 mL/kg. Intact pegnivacogin was a recently manufactured batch of API meeting all release testing specifications (purity = 93%). Expired pegnivacogin was an old lot of DP that had degraded over time, with a purity of 47% (by AX-HPLC), highly enriched in PEG degradants (>25-fold compared with CTMs used in the RADAR study).

Body temperature, blood pressure (systolic, diastolic, and mean arterial), heart rate, and electrocardiographic parameters (QRS duration and the RR, PR, and QT intervals) were monitored continuously from at least 2 hours before dosing until at least 24 hours postdose via telemetry, and data averaged in 15-minute increments for presentation.

Observations for morbidity, mortality, and injury were conducted at least twice daily for all animals. Clinical observations were conducted before dosing, at approximately 5 minutes and 6 hours postdose, and after completion of the cardiovascular monitoring period. Body weights were measured and recorded on the day before each dose administration. Blood samples for clinical pathology evaluations were collected pretest and on days 3, 5, 15, and 17. Blood samples for measurement of histamine level, complement factors (Bb fragment, C3a, C4a, CH50), and cytokine concentrations (IFN-γ, TNF-α, MIP-1α, MCP-1, and IL-6) were collected before and 5 minutes and 6 hours postdosing on days 1, 3, and 15.

Complement and cytokine analyses were conducted by the laboratory of Dr Patsy Giclas at the National Jewish Health Complement Laboratory per its standard procedures. Complement factors were measured in ELISA assays, CH50 in a standard sheep red blood cell hemolytic assay, and cytokines by Luminex. Sample values measured as less than LLOQ are reported as LLOQ/2 for calculation of means and SDs. All other laboratory measures were performed at MPI per its standard procedures.

No adverse effects related to the administration of intact or expired pegnivacogin were observed in this study. A subset of physiologic data collected via telemetry (body temperature, heart rate, and mean arterial pressure) is presented in Fig E3. No changes compared with control treatment were observed in response to treatment with pegnivacogin. Lower mean arterial pressure readings (Fig E3, C) in the expired pegnivacogin treatment group are due to 3 of 6 animals having lower pressure reading before dosing on day 15, so are not attributable to the test article. Spikes in recordings bracketing the periods at approximately 5 minutes and 6 hours postdose are due to sample collection.

Cytokine and histamine measurements are provided in Fig E4. No changes compared with control treatment were observed in response to treatment with pegnivacogin. Three of the cytokines (TNF-α, MIP-1α, and IL-6) were essentially at the LLOQ for all time points and treatments. In addition, no measures fell outside the normal range for individual animals in either treatment or control group.

Complement measurements are provided in Fig E5. As with cytokine and histamine measures, no changes compared with control treatment were observed in response to treatment with pegnivacogin, and no individual measures fell outside the normal range in either treatment or control group.

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FIG E1. Ability of anti-PEG antibody-positive QC to recognize molecular species relevant to the RADAR trial. 

A, ELISAs. Plates were coated with 3 μg per well of the indicated antigens, washed, and then incubated with duplicate aliquots of human plasma samples previously characterized as negative (designated control plasma and –QC) or positive for anti-PEG antibody (designated 213-10, + QC, and + standard).

B, Competition ELISAs. Duplicate aliquots of the positive QC were incubated with 0 to 20 μg of the competing antigens indicated in the figure legend, then carried through the antipegloticase ELISA. Antigens tested are defined in the Methods section; uricase is the un-PEGylated recombinant porcine urate oxidase used in pegloticase.
FIG E2. Determination of anti-PEG antibody isotype and IgG subclass. The samples tested are from the 3 patients who experienced first-exposure serious allergic-like reactions to pegnivacogin (IDs shown in figure legend).
FIG E3. Physiologic response of cynomolgus monkeys to pegnivacogin treatment. A, Body temperature. B, Heart rate. C, Mean arterial pressure. Spikes in readouts bracketing the 5-minute and 6-hour time period are sample collection times. N = 6 for all measures.
FIG E4. Cytokine and histamine response of cynomolgus monkeys to pegnivacogin treatment. A, TNF-α. B, IFN-γ. C, MCP-1. D, MIP-1α. E, IL-6. F, Histamine. Data presented as mean ± SD. N = 6 for all measures.
FIG E5. Complement response of cynomolgus monkeys to pegnivacogin treatment. A, C3a. B, C4a. C, Bb fragment. D, CH50 activity. Data presented as mean ± SD. N = 6 for all measures.