Letter to the Editor

Pre-existing anti–polyethylene glycol antibody linked to first-exposure allergic reactions to pegnivacogin, a PEGylated RNA aptamer

To the Editor:

Nucleic acid aptamers are a novel class of drugs that can be selected to inhibit targets of interest, including protein-protein interactions.[1] Pegnivacogin is a 2′-fluoropyrimidine–modified RNA aptamer that inhibits coagulation factor IXa, coupled to an approximately 40-kDa branched molecule of methoxypolyethylene glycol (mPEG), to increase its concentration and half-life in plasma.[2] During the RADAR[3] phase 2b clinical trial in patients with acute coronary syndrome, allergic reactions occurred within minutes of a first dose of pegnivacogin in 3 of 640 patients (Table I). Two met criteria for anaphylaxis, and 1 was an isolated dermal reaction; each event was deemed serious, and 1 life-threatening, and together they led to early termination of the trial.[3]

In a broad investigation into a cause for these 3 events (detailed in Methods in this article's Online Repository at www.jacionline.org), a clinical database review found no other serious allergic reactions (SARs) to pegnivacogin; a quality analysis found no aggregation, degradation, or other deviations of the study product from specifications; and a primate pharmacology study found no evidence that pegnivacogin caused an inflammatory response, histamine release, or complement activation. However, blinded testing of more than half of all RADAR patients identified an association between high levels of antibody to polyethylene glycol (PEG) and the first-exposure allergic reactions. In addition to the immediate relevance, our findings are the first to document the potential clinical significance of pre-existing antibody to PEG, a component of numerous consumer and medicinal products.

Initially, coded samples from 31 RADAR patients were tested for anti-PEG antibody (see Analytical methods and Fig E1 in this article's Online Repository at www.jacionline.org) in 2 ELISAs to detect IgG binding to pegloticase, a PEGylated urate oxidase (a protein not expressed in humans), and to the 40-kDa mPEG component of pegnivacogin. Unblinding of the data revealed that samples giving the highest signals in both ELISAs were from the 3 patients with SARs (predose from patients 418-008 and 406-003; 88-day postinfusion from patient 602-004 from whom no predose sample was available). Direct (Fig 1, *A*) and competition ELISAs (Fig 1, *B*) showed that antibody from each patient could bind to linear and branched PEGs of 5 to 40 kDa, presented as free mPEG or PEG-diol (lacking methoxy termini), or when conjugated via different linkages to 2 proteins and pegnivacogin; importantly, they did not recognize the un-PEGylated aptamer. The predominant heavy-chain isotype was IgG in all 3 patients, and for patient 418-008, who had the highest anti-PEG antibody level, IgG_2 was the predominant IgG subtype (see Fig E2 in this article's Online Repository available at www.jacionline.org).

To assess the overall prevalence and levels of pre-existing anti-PEG antibody, the sponsor submitted coded pretreatment plasma samples from 328 other RADAR patients who had not experienced allergic reactions (all remaining samples available). Recoded samples available from 26 of the initial 31 patients were added, to give a set of 354 unique patient samples (353 pretreatment and 1 posttreatment). In the pegloticase ELISA, 139 of these exceeded the A405 cutoff point of 0.15, with about a third having an A405 value of less than 0.2. Ninety samples with an A405 value of more than 0.2 were then retested with a validated automated competition ELISA, in which pegloticase was the plate coating and 10-kDa PEG-diol was the competitor.[4] Of these, 82 retested as positive with confirmed specificity for PEG (Fig 1, *C*). Applying this false-positive rate of 9%, we estimate that 36% of all RADAR patients tested were positive for anti-PEG antibody, with 23% having an A405 value of more than 0.2 in the pegloticase ELISA. The 3 patients with SARs were among only 8 with an A405 value of 1.0 or more (top 2.3%), with patient 418-008 having the highest A405 value of 1.9. Two of these 8 patients received heparin in the trial; of the other 6, 3 (50%) experienced allergic SARs. In further testing of 15 samples with A405 values within the 90th percentile, anti-PEG IgG titers for the 3 patients with SARs ranged from 1:120 to 1:3100 (patient 418-008), compared with approximately 1:110 to 1:950 for the 12 patients without allergies.

Oligonucleotides can potentially trigger allergic-like events by activating the alternative complement pathway, or an innate immune response via Toll-like receptors.[5] No evidence of the former mechanism was observed with pegnivacogin in primate studies (see Figs E3-E5 in this article's Online Repository at www.jacionline.org) or in phase 1 patients across a wide range of doses. Activation of Toll-like receptors typically elicits a mitogenic response with flu-like symptoms, which were not observed with the reactions in RADAR. Antibody-mediated mechanisms can also trigger allergy, but humans are not generally exposed to RNA oligonucleotides, and antibody to bacterial DNA and antinuclear autoantibodies do not react significantly with RNA. In contrast, free PEGs are used as additives in numerous consumer products and topical and parenteral medications, and

TABLE I. Subjects experiencing SARs in RADAR

Patient ID	Onset (min)	Symptoms	Treatment	Resolution (h)	Allergy history
602-004	5	GI, D, P, H	IVF, H1, IVV, S	6	2 mo prior diffuse urticaria to unknown agent
406-003	25	D	S, H1, H2	0.4	Contrast dye, hay fever
418-008	5	P, D, H	S, H1, H2, IVF, IVV, I, Inh	1144	Recent allergy to β-blockers and steroids

All 3 patients were female; 602-004 was treated at a site in Poland and the other 2 patients were treated in Germany. For additional information regarding these patients, see this article's REG1-CLIN211a section in the Online Repository at www.jacionline.org. *Note:* As substantially more information is available on these 3 subjects than for other trial participants, inferences regarding the possible role of sex, geography, or allergic history are cautioned against.

D, Dermal; *GI*, gastrointestinal; *H*, hypotension; *H1*, H1 blocker; *H2*, H2 blocker; *I*, intubation; *Inh*, inhalers; *IVV*, intravenous vasopressors; *IVF*, intravenous fluid resuscitation; *P*, pulmonary; *S*, steroids.

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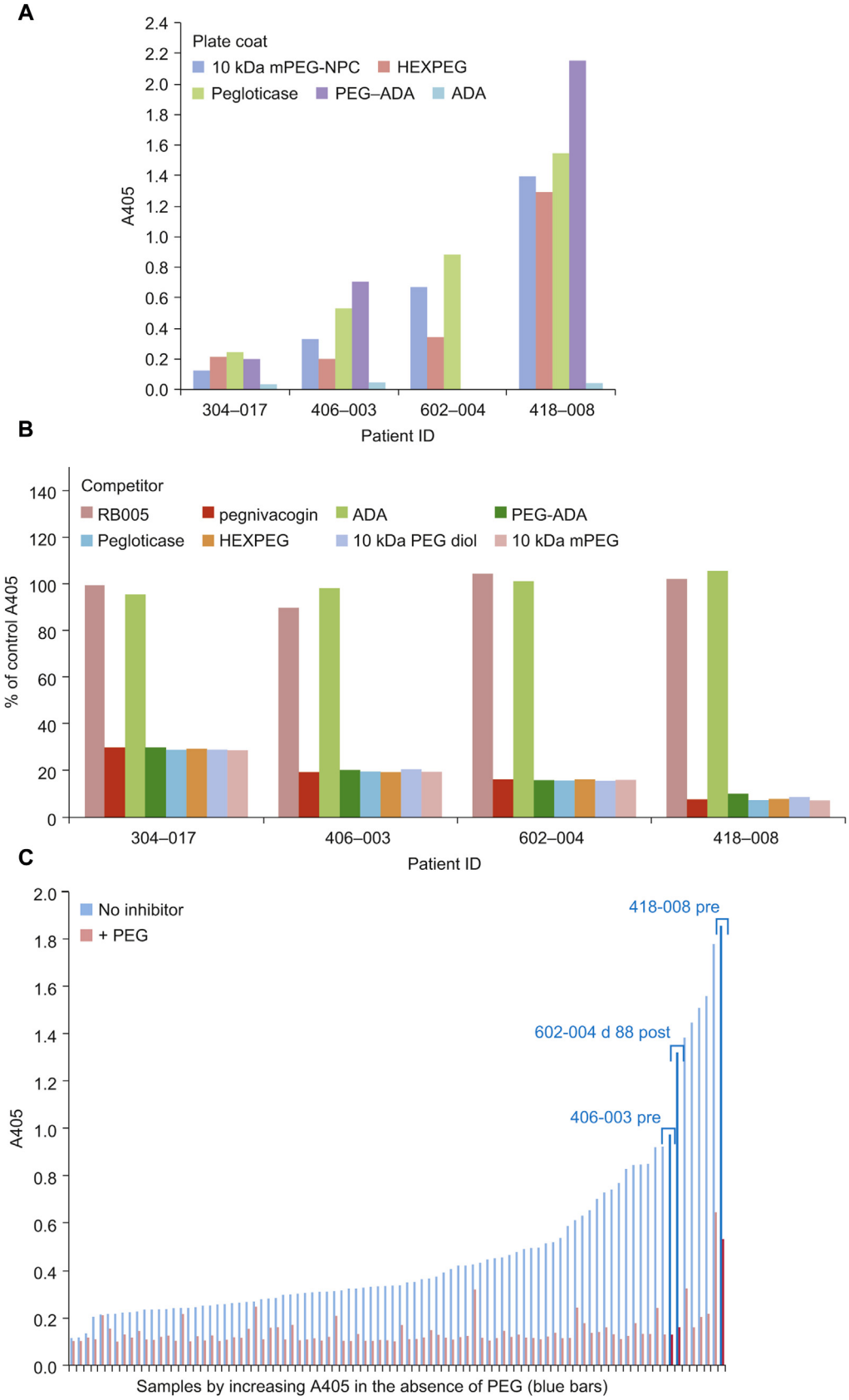
A
Plate coat
10 kDa mPEG-NPC
HEXPEG
Pegloticase
PEG–ADA
ADA
A405
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
304–017
406–003
602–004
418–008
Patient ID
B
Competitor
RB005
pegnivacogin
ADA
PEG-ADA
Pegloticase
HEXPEG
10 kDa PEG diol
10 kDa mPEG
% of control A405
140
120
100
80
60
40
20
0
304–017
406–003
602–004
418–008
Patient ID
C
No inhibitor
+ PEG
418-008 pre
602-004 d 88 post
406-003 pre
A405
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Samples by increasing A405 in the absence of PEG (blue bars)

as osmotic laxatives. Perhaps as a result of this wide exposure, anti-PEG antibodies, of no apparent clinical significance, have been detected by various methods as an incidental finding in several study populations at a prevalence of approximately 3% to more than 40%.[6] mPEGs have also been used for more than 25 years to covalently modify (PEGylate) biologically active molecules as a means of masking epitopes and slowing elimination. Some PEGylated enzymes and liposomes have recently been found to induce anti-PEG antibodies.[6] Findings in clinical trials of PEGylated enzymes support a role for high levels of pre-existing anti-PEG antibodies in causing first-exposure reactions to pegnivacogin.

In a phase 2 trial of pegloticase for refractory gout, we found anti-PEG antibodies in pretreatment samples of 5 of 27 (19%) patients. The patient with the highest level had an adverse reaction during his first infusion of pegloticase, and patients in whom high levels of anti-PEG antibodies were induced by pegloticase had more frequent and more severe infusion reactions than did those who remained antibody negative.[4] Infusion reactions, including anaphylaxis-like SAEs, were also associated with higher levels of pegloticase-induced anti-PEG antibodies during phase 3 investigation.[7] In a phase 1 trial for treating phenylketonuria, a PEGylated phenylalanine ammonia lyase (rAvPAL-PEG) induced anti-PEG antibodies in all 25 participants, 2 of whom had hypersensitivity reactions to an incidentally prescribed birth control medication formulated with PEG before its use was forbidden.[8]

We postulate that a high level of pre-existing anti-PEG antibodies was a major, but not the sole, factor necessary for triggering first-exposure allergic reaction to pegnivacogin. A likely second factor is the relatively large amount of PEG delivered in a single 1 mg/kg bolus dose of pegnivacogin, for example, approximately 64 mg of PEG for an 80-kg patient. In contrast, with peginterferon alfa2a (PEGASYS), which also contains a single 40-kDa branched mPEG, a standard dose would deliver about 0.12 mg of PEG. Other factors must also be involved, as a few other RADAR patients with similar high levels of pre-existing anti-PEG antibodies did not experience an allergic reaction to pegnivacogin. This could reflect differences in properties of anti-PEG antibodies in individual patients, as well as differences in susceptibility to antibody-triggered allergic reactions, that is, the same factors that underlie the spectrum of responses that antidrug antibodies elicit to some antibiotics and un-PEGylated protein-based biologics.[9,10]

On the basis of our findings, we advise testing for pre-existing anti-PEG antibodies during clinical trials of new PEGylated therapeutic agents, particularly those containing high densities of PEG, or that are given at doses that would expose patients to relatively large amounts of PEG. Further research is needed into the prevalence of "incidental" anti-PEG antibodies, the basis for PEG immunogenicity, and the mechanism(s) by which PEG-anti-PEG immune complexes trigger allergic reactions.

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FIG 1. Characterization of anti-PEG antibodies in RADAR patient samples. **A,** ELISAs to assess binding to the antigens indicated in the figure (for description and source of these materials, see this article's Online Repository at www.jacionline.org). **B,** Competition ELISA. Duplicate aliquots of samples, one spiked with 20 μg of the indicated competing antigen, were tested in the antipegloticase ELISA. IDs 406-003, 602-004, and 418-008 are the 3 patients who experienced SARs to pegnivacogin; ID 304-017 had no reaction to pegnivacogin, but was positive for anti-PEG antibody. **C,** Automated competition ELISA. The 90 samples tested had an A405 value of more than 0.2 in the antipegloticase ELISA (see text for details). *Blue and red bars* are, respectively, the A405 obtained in the absence and presence of competing antigen (10 kDa PEG-diol). The *darker blue and red bars* with sample IDs indicate the 3 patients who experienced SARs to pegnivacogin.

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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 (Bridgewater, 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.[E1] 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.[E1] 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.[E2] 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,[E1] 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 IgG_1-, IgG_2-, IgG_3-, and IgG_4-specific antibodies (all from Sigma). Biotinylated antibodies were detected with the Vectastain ABC Kit AK-5000 (Vector Laboratories, Burlingame, Calif). Fig E2 shows results of the determinations of IgM and IgG isotype and IgG subclass.

REG1-CLIN211a. REG1-CLIN211a was conducted as a follow-up to REG1-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[E3] 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 REG1 (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 pengivacogin 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, MIP1-α, 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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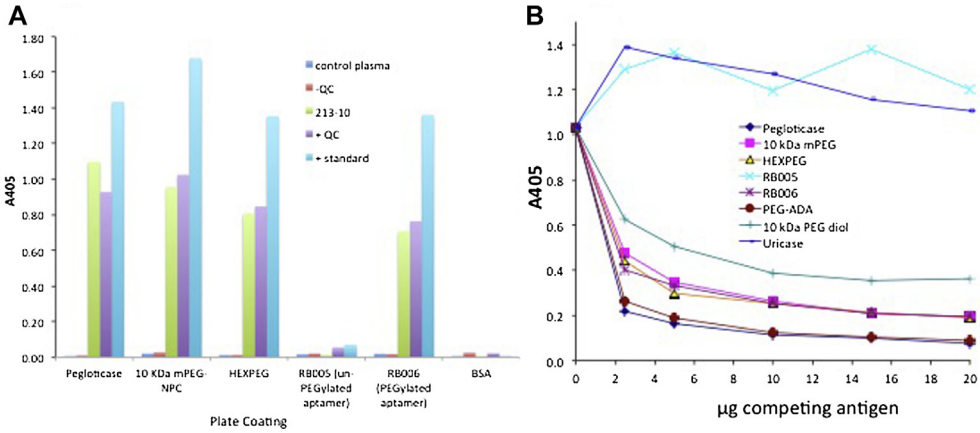

FIG E1. Ability of anti-PEG antibody-positive QC to recognize molecular species relevant to the RADAR trial. **A,** ELISAs. Plates were coated with 3 μg per well of the indicated antigens, washed, and then incubated with duplicate aliquots of human plasma samples previously characterized as negative (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.

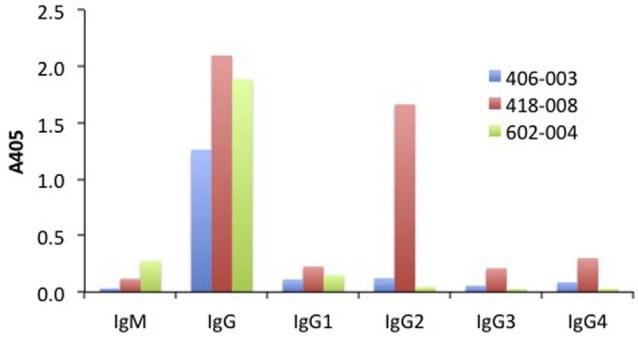

FIG E2. Determination of anti-PEG antibody isotype and IgG subclass. The samples tested are from the 3 patients who experienced first-exposure serious allergic-like reactions to pegnivacogin (IDs shown in figure legend).

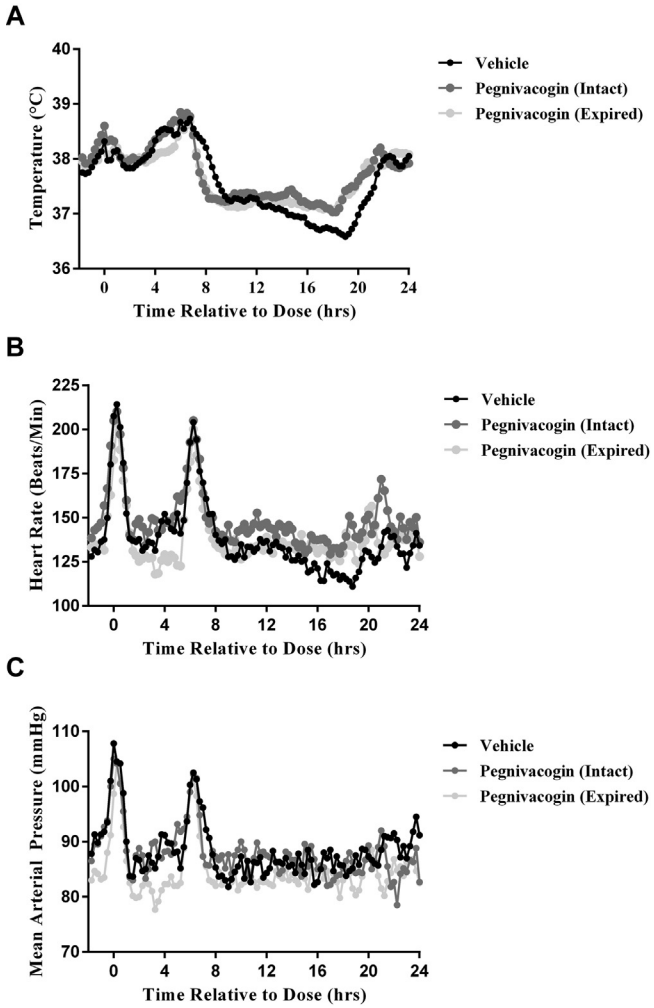

FIG E3. Physiologic response of cynomolgus monkeys to pegnivacogin treatment. **A,** Body temperature. **B,** Heart rate. **C,** Mean arterial pressure. Spikes in readouts bracketing the 5-minute and 6-hour time period are sample collection times. N = 6 for all measures.

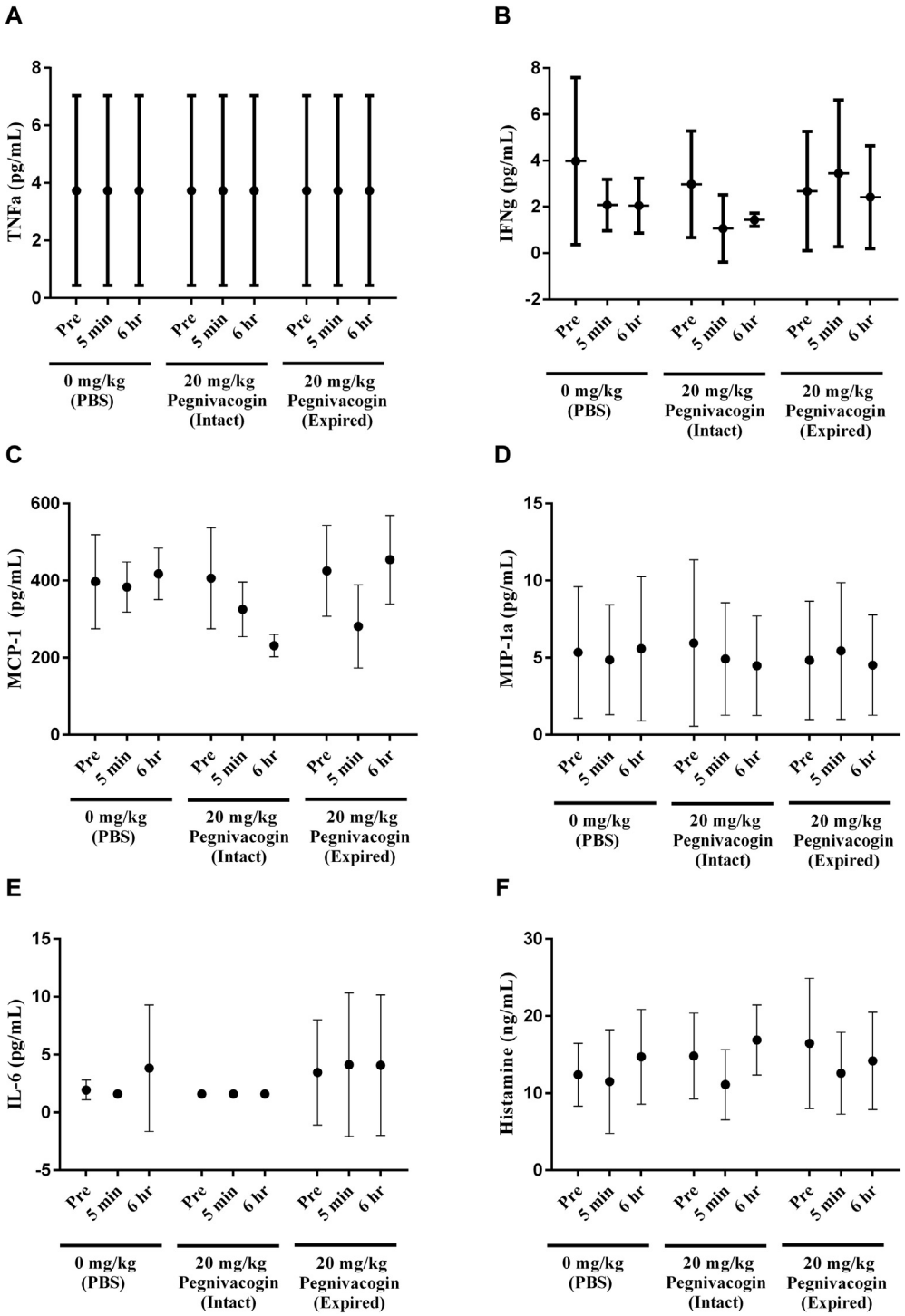

FIG E4. Cytokine and histamine response of cynomolgus monkeys to pegnivacogin treatment. **A,** TNF-α. **B,** IFN-γ. **C,** MCP-1. **D,** MIP-1α. **E,** IL-6. **F,** Histamine. Data presented as mean ± SD. N = 6 for all measures.

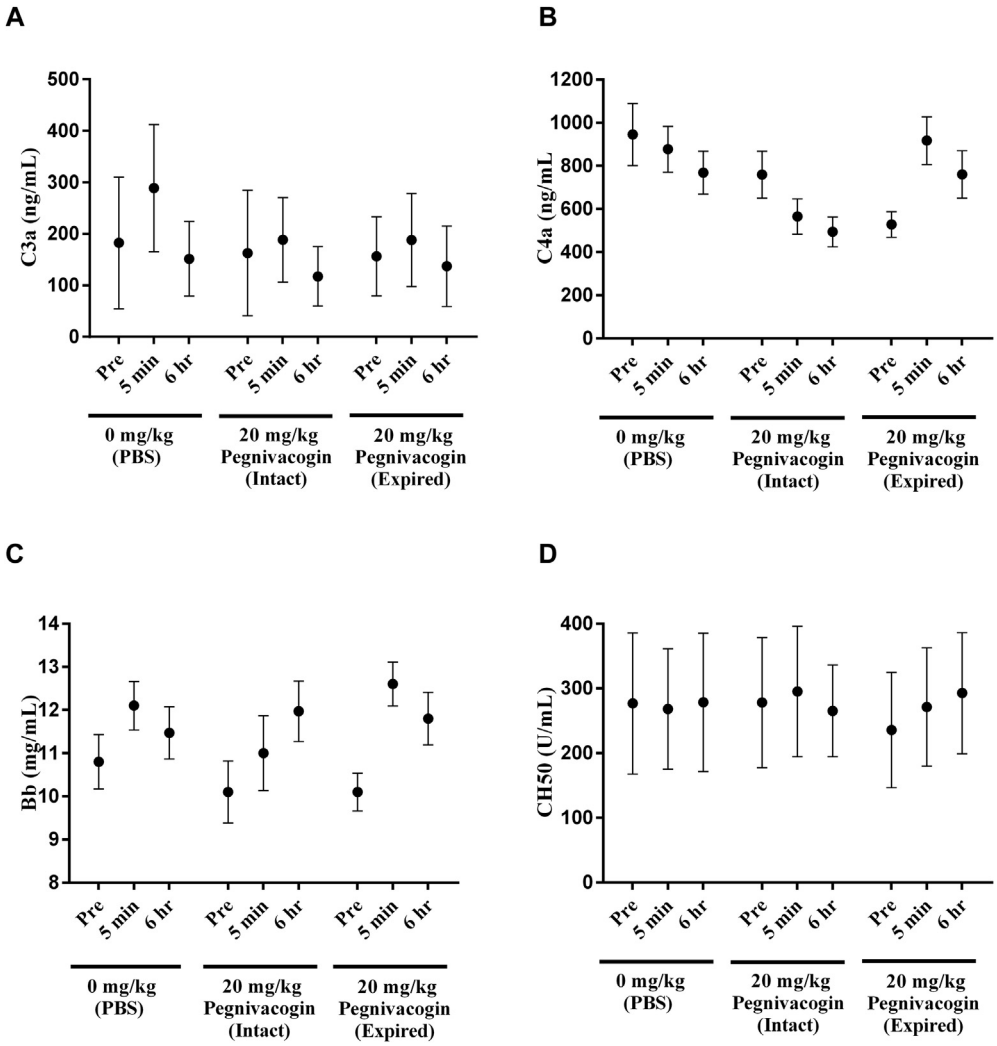

FIG E5. Complement response of cynomolgus monkeys to pegnivacogin treatment. **A,** C3a. **B,** C4a. **C,** Bb fragment. **D,** CH50 activity. Data presented as mean ± SD. N = 6 for all measures.