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Rapid Communication

Subvisible Particle Content, Formulation, and Dose of an Erythropoietin Peptide Mimetic Product Are Associated With Severe Adverse Postmarketing Events

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ABSTRACT

Peginesatide (Omontys®; Affymax, Inc., Cupertino, CA) was voluntarily withdrawn from the market less than a year after the product launch. Although clinical trials had demonstrated the drug to be safe and efficacious, 49 cases of anaphylaxis, including 7 fatalities, were reported not long after market introduction. Commercialization was initiated with a multiuse vial presentation, which differs in formulation from the single-use vial presentation used in phase 3 studies. Standard physical and chemical testing did not indicate any deviation from product specifications in either formulation. However, an analysis of subvisible particulates using nanoparticle tracking analysis and flow imaging revealed a significantly higher concentration of subvisible particles in the multiuse vial presentation linked to the hypersensitivity cases. Although it is unknown whether the elevated particulate content is causally related to these serious adverse events, this report illustrates the utility of characterizing subvisible particulates not captured by conventional light obscuration.

Introduction

The biopharmaceutical industry has capitalized on techniques such as random phage display peptide libraries and affinity purification methods to develop the next generation of therapeutics. These biobetters may have little or no amino acid sequence homology to the original molecule, making extrapolation of their safety profile from prior experience uncertain. Peginesatide (Omontys®; Affymax, Inc., Cupertino, CA) is one such product. An approved erythropoiesis-stimulating agent (ESA), it is comprised of a synthetic erythropoietin (Epo) peptide mimetic covalently dimerized and linked to polyethylene glycol (PEG). Peginesatide has no amino acid homology to Epo but shares a short Epo receptor (EpoR)-binding motif with other Epo peptide mimetics.[1] Remarkably, this drug is an effective ESA in patients with pure red cell aplasia who have anti-Epo antibodies.[2] Approved by the US Food and Drug Administration (FDA) in March 2012, it was voluntarily withdrawn from the market on February 23, 2013, following an unexpected rise in severe adverse events and associated fatalities on first drug exposure.

An FDA wide task force analyzed these adverse events using both data supplied by the drug manufacturer and generated by the agency. The FDA identified 49 related cases of anaphylaxis (including

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7 fatalities) based on the National Institute of Allergy and Infectious Diseases consensus case definition,[3] yielding an anaphylaxis rate of 1.8 per 1000 exposed patients, which is similar to the rate recently reported in this population.[4] The corresponding hypersensitivity rate was 3.5 cases per 1000, a rate that is considerably higher than the premarket clinical trials' experience of 0.84 hypersensitivity cases per 1000 exposed patients (with no fatalities), which is similar to the postmarket rate reported to the FDA for epoetin alfa.

As postmarket safety reports generally do not include as much information as the safety reports collected during clinical trials, analysis of the potential risk factors for anaphylaxis and death was limited. Prior ESA exposure was reported in 30 of 32 anaphylaxis cases (information was not available for the other 17 anaphylaxis cases), suggesting that these events are unique to peginesatide and not an ESA class effect. There was neither apparent geographic or demographic association nor a clear association with a particular drug lot. However, in 90% of the evaluable cases identified by the FDA, anaphylaxis occurred within 10 min of first drug exposure, and most fatal cases lacked the typical clinical manifestations associated with IgE-mediated type I hypersensitivity. Although most patients received a dose $\leq$10 mg, 4 of the 7 fatalities were associated with a dose >10 mg. Three of the severe cases ($n = 17$) had penicillin sensitivity, but no other drug sensitivities occurred in more than 1 severe case, and none of the reported fatalities shared a drug allergy. However, comorbidities such as left ventricular dysfunction, respiratory disorders, and hypertension were frequent and severe among the fatal cases.

The observed disparity in the pre- and postmarketing incidence of anaphylaxis focused attention on possible product quality differences between the drug product used during clinical trials and that introduced into the market. Peginesatide was manufactured and approved as both a single-use vial (SUV) and a multiuse vial (MUV), which differed in their formulation. Clinical trials primarily used the SUV formulation, but only the MUV formulation was marketed. Although the constituents of the MUV formulation are all generally recognized as safe, formulation composition is widely understood as having the potential to alter the properties of biological therapeutics, including the subvisible particulate (SVP) profile. SVPs are known to impact immunogenicity and have also been shown to promote inflammation.[5] Although control of SVPs in therapeutics has focused on particles $\geq$10 microns, numerous studies have shown that the overwhelming majority of SVPs are <10 microns. Advanced methods have been developed to provide morphological information for particle sizes in the micron range (e.g., flow imaging) and to quantify SVP in the submicron size range (e.g., nanoparticle tracking analysis [NTA]). These emerging techniques currently lack validated protocols, but their enhanced capacity to characterize and quantitate SVP may yield valuable insights into changes in product quality. For an in-depth discussion of the strengths and limitations of the techniques, see the reviews by Zolls, Narhi, and Filipe et al.[6-8]

As part of its investigation, the Task Force also reviewed batch records and Certificates of Analysis for all released lots, inspected drug manufacturing sites, and conducted release and extended characterization testing of undistributed MUV, as well as MUV from clinics where serious adverse events had occurred. In addition, experimental studies on the biological activities of peginesatide were performed by FDA laboratories and their consultants at the National Institutes of Health. Here we report, in part, the results of those investigations.

Results and Discussion

Product Quality Release Testing

After withdrawal of peginesatide (CAS #913976-27-9) from the market, extensive testing of MUV samples was independently conducted by the FDA and the manufacturer. All testing confirmed that the drug product met approved release specifications, including United States Pharmacopeia (USP) <788> limits for SVP (proprietary data not shown). Furthermore, the results were comparable to analysis of lots performed at the time of release, and no new impurities or contaminates were identified. Similarly, the results of limited FDA testing of unopened MUV returned from clinics where adverse events had occurred met the release specifications for content and impurities.

Extended Characterization of SVPs

Particle concentrations within the size range of 50 nm-1 μm were evaluated using NTA. For particles >100 nm, significantly higher concentrations were found in MUV lots compared with SUV lots (Table 1). Additionally, a significant difference ($p < 0.05$) in particle concentration was found between MUV lots C18881 and C19258 (Fig. 1, MUV-A) versus MUV lots C18686 and C18696 (Fig. 1, MUV-B). As revealed in Figure 1, the differences in particle concentration between MUV and SUV lots are largely attributable to the MUV-A lots. A similar trend among these MUV lots was also observed for Z average and polydispersity index by dynamic light scattering (see Supplementary Data and Table S1). The particulate content of the SUV lots examined was found to be independent of product concentration and product lot.

Flow imaging used to evaluate particles in the micron range captured mostly small and spherical images resembling silicon oil or air, particularly in the SUV samples. Thus, results were digitally filtered using particle libraries constructed to exclude such images and those of insufficient resolution (see Supplementary Data and Table S2 and Fig. S1). After applying these filters, the average particle concentration in MUV lots was dramatically higher than in SUV lots (Table 2). Although the detected concentration of particles >10 microns was low (<1000 particles/mL) compared with USP <788> limits for the light obscuration method, comparison of particle concentrations in SUV and MUV samples revealed significant ($p < 0.001$) differences between them in all the measured size ranges (Fig. 2). Furthermore, in each size range, the distribution of particle concentrations was significantly different between MUV lots and SUV lots ($p < 0.01$ by Kolmogorov–Smirnov test).

The consistency of the analytical results indicates that significant differences exist between the particulate concentration of the SUV and MUV lots, with the latter containing more particulates and greater variability in particulate load. We infer that these differences are most likely because of the MUV formulation but have not identified the specific factor that may be responsible. Although all

Table 1
NTA Peginesatide Median Particle Concentrations[a,b]

Hydrodynamic Diameter (nm)	SUV	MUV	p[c]
50-1000	9763	29,934	0.028
50-100	1610	1197	0.673
101-200	5176	14,426	0.022
201-300	1155	8526	0.001
301-400	286	2068	0.001
401-500	88	519	0.001
501-600	30	237	0.001
601-700	8	102	0.003
701-800	3	48	0.002
801-900	2	30	0.002
901-1000	2	18	0.008

[a] Particles/mL ($\times 10^4$).
[b] SUV and MUV were independently measured 6 (each SUV lot in duplicate) and 12 (each MUV lot in triplicate) times, respectively.
[c] Mann–Whitney test.

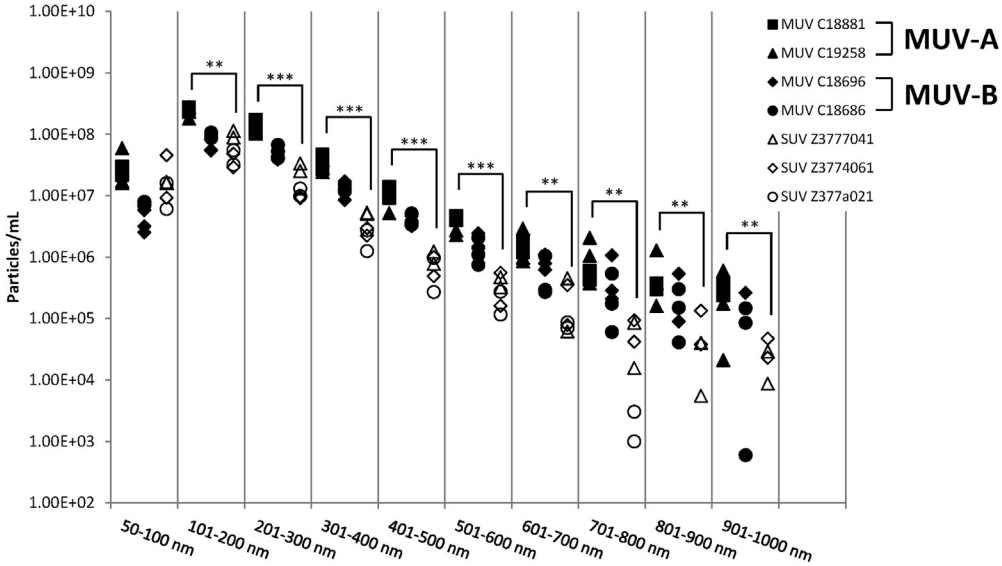

Figure 1. Size distribution and concentration of peginesatide particulates 50-1000 nm. On analysis, MUV lots segregated into 2 groups (MUV-A and MUV-B; see text). MUV lots C18881 and C19258 (MUV-A) had significantly higher particle loads than SUV lots. Kruskal–Wallis tests were performed for each size range shown along the *x*-axis, followed by Dunn's multiple comparison test for SUV with MUV-A and SUV with MUV-B; $n = 6$ for each group; $^{**}p < 0.01$, $^{***}p < 0.001$.

peginesatide samples passed USP <788> limits for particulates, our results suggest that more sensitive analytical monitoring of SVP could distinguish differences in product characteristics that would otherwise go undetected.

Human Mast Cell Activation

As the EpoR is known to be widely expressed outside the erythroid lineage and mast cells in mice are reported to be responsive to Epo, the possibility that peginesatide could directly induce or promote the release of anaphylactic mediators from human mast cells (HuMCs) was addressed.[9] The MUV product was incubated under the indicated conditions with HuMCs, and mast cell activation was measured as a function of β-hexosaminidase (β-hex) release (Fig. S2a) and prostaglandin D_2 (PGD2) production (Fig. S2b). Peginesatide did not directly induce mast cell degranulation (β-hex release) or PGD2 production, nor did it enhance mast cell degranulation or PGD2 production in cells activated through IgE receptor cross-linking.

As many individuals have been reported to have circulating antibodies to PEG, we also addressed whether such anti-PEG antibodies could cross-link peginesatide through its PEG moiety and thus trigger basophil activation.[10,11] Preliminary results indicate that peginesatide does not directly activate primary human basophils as measured by $CD63^+$ $CD203c^+$ expression, nor do anti-PEG antibodies have any impact on activation of basophils preincubated with peginesatide (data not shown). We conclude that peginesatide does not induce or enhance the activation of human basophils or mast cells. This conclusion is based on the testing of a limited number of healthy donors, and we cannot exclude the possibility that peginesatide signaling through one of the described EpoR polymorphs could activate basophils and/or mast cells.[12] However, incubation of peginesatide did reproducibly induce release of IL-8 in a whole blood assay, but the relevance of this finding remains under investigation (personal communication, Z. Zhou and S. Kozlowski).

Table 2
Normalized FlowCAM Peginesatide Particle Concentrations[a]

ESD (μm)	SUV			MUV			
	Z377a021	Z3777041	Z3774061	C18686	C18696	C18881	C19258
10-15	1.00	0.40	0.54	2.24	3.28	2.28	3.23
15-20	1.00	0.76	0.48	3.13	4.29	3.11	5.49
20-25	1.00	0.49	0.43	4.05	4.73	4.54	7.28
≥25	1.00	0.82	1.92	13.60	9.02	14.26	24.69

[a] Mean particle concentrations normalized to SUV lot Z377a021 (see Materials and Methods section). Each lot was independently measured 5-10 times.

Conclusions

Postmarketing anaphylaxis was observed after administration of the peginesatide MUV presentation, with a disproportionate number of fatal cases occurring at higher doses. Although the biological mechanism underlying the observed hypersensitivity events remains under investigation,[13] we found that peginesatide did not directly induce HuMC activation *ex vivo* at concentrations up to 100X greater than that found *in vivo* at the highest administered dose. Despite passing all release tests, including USP <788>, using more sensitive techniques to quantitate subvisible particles revealed a significantly different particulate profile in the MUV compared with the SUV product. This case illustrates the potential value of using methods beyond those described in USP <788> to characterize SVPs in biological therapeutics.

Materials and Methods

Samples and Reagents

Samples and formulation buffers were obtained directly from the manufacturer, distribution sites, or from the field by FDA investigators. The concentration of MUV lots (C18686, C18696, C18881, and C19258) was 10 mg/mL. The 3 available SUV lots were at 3 different concentrations: 4 mg/mL (Z3774061), 10 mg/mL (Z377a021), and 12 mg/mL (Z3777041). Particulate content was analyzed neat. PCC-54 was obtained from Thermo Scientific (Rockford, IL). Anti-PEG IgG and IgM were from Marine

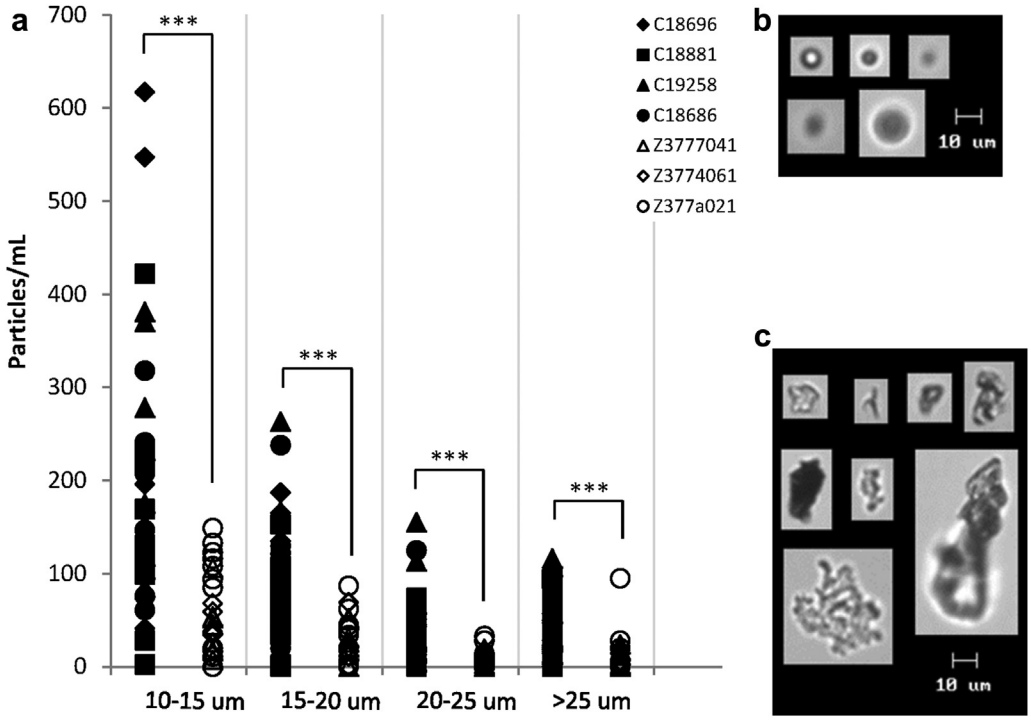

Figure 2. Size distribution and concentration of peginesatide particulates ≥10 μm. (a) Particle images detected via flow imaging were subject to global analysis (see Materials and Methods section). Statistically significant differences in particle concentration for different particle sizes were identified using Mann–Whitney tests performed for each size range shown along the *x*-axis. Sizes are reported as ESD. SUV (open symbols) and MUV (closed symbols) lots were imaged in 23 and 35 runs, respectively; ***$p < 0.001$. Examples of images (b) removed or (c) retained after global analysis filtration are shown (see Supplementary Data for complete image sets).

Biotechnology Services (Portland, ME) and ANP Technologies, Inc. (Newark, DE).

Nanoparticle Tracking Analysis

NTA was performed using a NanoSight NS500 system (Malvern) equipped with a 405-nm laser and an electron multiplying charge-coupled device high-sensitivity camera with 20X objective magnification. All measurements of SUV and MUV samples were performed without dilution under the same conditions as follows: for each sample, 3 movies were recorded at 22°C for 120 s with camera level at 9. The preprocessing settings were also the same for all samples: detection threshold of 24, minimal track length of 10, minimal expected size of 30 nm, and blur of 3 × 3.

Flow Imaging Analysis

Samples were imaged using the FlowCAM VS1 system (Fluid Imaging Technologies, Inc., Scarborough, ME) and analyzed using VisualSpreadsheet software v. 3.0.3 (Fluid Imaging Technologies, Inc.). For each measurement, 450 μL of sample was run through an 80-μm FC80FV flow cell and observed through a 10X objective after manually priming with deionized water. Between measurements, the system was flushed with 10% PCC-54 detergent and then 0.2-μm filtered, 18.2-mΩ-cm deionized water was measured to ensure that cross-contamination had not occurred.

Because of the limited availability of materials, the FlowCAM protocol was optimized in parallel with data collection, which occurred over 3 different experimental series (experiments 1-3). A global analysis of all results and individual analyses optimized for each experimental series were performed. Sizes reported are equivalent spherical diameter. The images were processed by excluding particles with equivalent spherical diameter <10 μm, edge gradient <5, or >1 particle per chain. For the global analysis, images were collected using a threshold value of 8 for dark and light pixels, 3 close hole iterations, and a 2- or 4-μm distance to nearest neighbor setting. Libraries were created by selecting images that resembled air, silicon oil, or were of insufficient resolution; these libraries were then used to filter out statistically similar images. The analysis was also performed separately for each experimental series to account for changes made during concurrent method development, which included optimization of focusing and image analysis. Raw images recorded in experiment 3 were recorded and reanalyzed under conditions better suited for morphology characterization (threshold values of 15 dark pixels and 50 light pixels, 3 close hole iterations, and a 4-μm distance to nearest neighbor). Images were filtered using libraries specific to each experiment. To limit the impact of changes made to the protocol, these results were normalized to SUV lot Z377a021 within each experiment and then averaged.

Statistical Analysis

Particulate concentrations of SUV and MUV lots were compared using 2-tailed Mann–Whitney test. For comparisons of SUV, MUV-A, and MUV-B, Kruskal–Wallis tests were performed, with Dunn's multiple comparison test applied to SUV versus both MUV-A and MUV-B groups. Distributions of MUV and SUV particle concentrations for each size range determined via fluid imaging analysis were compared using a Kolmogorov–Smirnov test.

HuMC Cultures

Pluripotent $CD34^+$ progenitor cells from healthy volunteers were enriched after leukapheresis of peripheral blood (National Institutes of Health Institutional Review Board protocol #98-I-0027) and

differentiated into HuMCs as described previously.[14] The cells used in the degranulation and PGD2 release assays were greater than 99% HuMCs.[15]

HuMC Degranulation and Prostaglandin D_2 Release Assays

HuMCs were sensitized overnight using 100 ng/mL biotinylated human IgE.[16] The HuMCs were then incubated with different concentrations of peginesatide from MUV in the presence or the absence of streptavidin (SA). β-hex activity in the cell supernatants and lysates was measured and degranulation defined as the percentage of the total β-hex recovered from the supernatants.[17] For prostaglandin D_2 measurements, the HuMCs were sensitized overnight as described previously and stimulated with different concentrations of peginesatide (MUV) and in the presence or the absence of SA, 100 ng/mL stem cell factor, or both for 30 min at 37°C, and the cell-free supernatants were collected. Prostaglandin D_2 release was detected using a prostaglandin D_2–MOX EIA kit (Cayman Chemical) according to manufacturer's instructions.

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References

1. Wrighton NC, Balasubramanian P, Barbone FP, et al. Increased potency of an erythropoietin peptide mimetic through covalent dimerization. *Nat Biotechnol*. 1997;15:1261-1265.
2. Macdougall IC, Rossert J, Casadevall N, et al. A peptide-based erythropoietin-receptor agonist for pure red-cell aplasia. *N Engl J Med*. 2009;361:1848-1855.
3. Sampson HA, Munoz-Furlong A, Campbell RL, et al. Second symposium on the definition and management of anaphylaxis: summary report—Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *J Allergy Clin Immunol*. 2006;117:391-397.
4. Bennett CL, Jacob S, Hymes J, Usvyat LA, Maddux FW. Anaphylaxis and hypotension after administration of peginesatide. *N Engl J Med*. 2014;370:2055-2056.
5. Joubert MK, Hokom M, Eakin C, et al. Highly aggregated antibody therapeutics can enhance the in vitro innate and late-stage T-cell immune responses. *J Biol Chem*. 2012;287:25266-25279.
6. Zolls S, Weinbuch D, Wiggenhorn M, et al. Flow imaging microscopy for protein particle analysis—a comparative evaluation of four different analytical instruments. *AAPS J*. 2013;15:1200-1211.
7. Narhi LO, Jiang Y, Cao S, Benedek K, Shnek D. A critical review of analytical methods for subvisible particles. *Curr Pharm Biotechnol*. 2009;10:373-381.
8. Filipe V, Hawe A, Jiskoot W. Critical evaluation of nanoparticle tracking analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm Res*. 2010;27:796-810.
9. Ribatti D, Crivellato E, Nico B, Guidolin D, Gassmann M, Djonov V. Mast cells and macrophages in duodenal mucosa of mice overexpressing erythropoietin. *J Anat*. 2009;215:548-554.
10. Liu Y, Reidler H, Pan J, et al. A double antigen bridging immunogenicity ELISA for the detection of antibodies to polyethylene glycol polymers. *J Pharmacol Toxicol Methods*. 2011;64:238-245.
11. Garay RP, El-Gewely R, Armstrong JK, Garratty G, Richette P. Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opin Drug Deliv*. 2012;9:1319-1323.
12. de la Chapelle A, Traskelin AL, Juvonen E. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. *Proc Natl Acad Sci U S A*. 1993;90:4495-4499.
13. Weaver JL, Boyne M, Pang E, Chimalakonda K, Howard KE. Nonclinical evaluation of the potential for mast cell activation by an erythropoietin analog. *Toxicol Appl Pharmacol*. 2015;287(3):246-252.
14. Radinger M, Jensen BM, Kuehn HS, Kirshenbaum A, Gilfillan AM. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. *Curr Protoc Immunol*. 2010;Chapter 7:Unit 7.37.
15. Kirshenbaum AS, Goff JP, Semere T, Foster B, Scott LM, Metcalfe DD. Demonstration that human mast cells arise from a progenitor cell population that is CD34+, c-kit +, and expresses aminopeptidase N (CD13). *Blood*. 1999;94:2333-2342.
16. Jensen BM, Beaven MA, Iwaki S, Metcalfe DD, Gilfillan AM. Concurrent inhibition of kit- and FcepsilonRI-mediated signaling: coordinated suppression of mast cell activation. *J Pharmacol Exp Ther*. 2008;324:128-138.
17. Woolhiser M, Okayama Y, Gilfillan A, Metcalfe D. IgG-dependent activation of human mast cells following up-regulation of FcgammaRI by IFN-gamma. *Eur J Immunol*. 2001;31:3298-3307.