Research Article

For reprint orders, please contact reprints@future-science.com

Bioanalysis

Anti-PEG antibody bioanalysis: a clinical case study with PEG-IFN-λ-1a and PEG-IFN-α2a in naive patients

Background: Extensive use of polyethylene glycol (PEG) in consumer products necessitates the assessment of anti-PEG antibodies (APAb). **Methods:** In clinical trials comparing PEG-IFN-λ to PEG-IFN-α, conventional bridge and direct assays were assessed. **Results & Conclusion:** The bridge assay detected IgM and IgG APAb reactive with common PEG sizes and derivatives at sufficient sensitivity, 15–500 ng/ml. Of subjects evaluated, 6% of PEG-IFN-λ and 9% of PEG-IFN-α subjects had persistent APAb while 60% of PEG-IFN-λ and 33% of PEG-IFN-α subjects had persistent anti-interferon antibodies (AIAb). Pre-existing APAb and AIAb prevalence was comparable (approximately 10% of subjects). APAb were earlier onset, less frequent, less persistent and lower titer than AIAb. No associated hypersensitivity events were reported.

Heather Myler*[,1], Matthew W Hruska[1], Subasree Srinivasan[1], Elizabeth Cooney[1], George Kong[1], Robert Dodge[1], Murli Krishna[1], Jie Zhu[2], Tonya Felix[3], Carol Gleason[1], Steven P Piccoli[1] & Binodh DeSilva[1]

[1]Bristol-Myers Squibb, Rt 206 & Provinceline Rd, Princeton NJ 08540, USA

[2]WuXi AppTec (Shanghai) Co., Ltd 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Shanghai 200131, China

[3]LabCorp, 115 Silvia Street, West Trenton, NJ 08628, USA

*Author for correspondence: heather.myler@bms.com

Most biotherapeutics elicit some level of antidrug antibody (ADA) response, and in most cases this immune response has little to no impact on the safety or efficacy of the biotherapeutics. However, in some cases ADA are associated with decreased exposure and loss of efficacy and in rare cases, ADA have been associated with serious adverse events [1,2].

The nature of these ADA responses can depend on several factors, including the type of therapeutic protein (e.g., for monoclonal antibodies, whether they are fully human, humanized, or chimeric), and/or the structure of the molecule. ADA directed at the components of the molecular structure, especially at the PEG portion of PEGylated proteins, has drawn considerable interest [3], and is included in the FDA guidance on the immunogenicity of therapeutic protein products [4,5]. Pegylation of therapeutic proteins and the potential consequences of ADA development directed at the PEG portion of the molecule have drawn attention due to expanded use of PEG, not only in prescription medicines, but also in nonprescription medicines, other consumer products and foods. This extensive use is thought to have led to a higher incidence of anti-PEG antibodies among the human population even though pegylation has been postulated to decrease the immunogenicity of proteins to which it is attached by increasing solubility, decreasing aggregation and potentially masking antigenic epitopes in the protein structure [6,7].

The best practices for measuring and characterizing anti-PEG Abs are still being debated. Significant investments in the field have been made including the implementation of a diverse set of bioanalytical techniques (e.g., surface plasmon resonance, direct ligand-binding assay [LBA], bridging LBA) [8] and the generation and characterization of numerous positive controls (PC; e.g., IgM, IgG and IgE isotyping controls, commercial and custom PC). Even with these investments, significant technical challenges still exist due to the repeating small motif structure of PEG, the relatively low affinity of anti-PEG Abs, the structural homology of PEG to detergents and the challenges of producing relevant PCs. Further, because of previously detected clinically relevant anti-PEG Abs of IgM

FUTURE SCIENCE part of fsg

and IgG isotypes [9–11], anti-PEG Ab assay formats should be able to measure a diverse set of isotypes, as with conventional ADA assay formats. However, it is important to note that there are several pegylated compounds that have been used in the treatment of various indications for over a decade and are still in use (e.g., PEG-IFN-α2a and pegfilgrastim), which have been shown to benefit patients in the absence of adverse events potentially related to PEG portions of the molecules. Only clinical evidence related to pegasparaginase and pegloticase demonstrates loss of efficacy and increased incidences of hypersensitivity reactions associated with measurable levels of anti-PEG antibodies [9–11].

In the clinical development program comparing a novel PEG-IFN-λ-1a (herein referred to as Lambda) to PEG-IFN-α (Alfa), it was of interest to understand the presence/absence of anti-PEG Abs. Interferon (IFN) Lambda is a member of the Type III IFN family whose biological characteristics are similar to those of Type I IFNs, such as IFNα and IFNβ [12,13]. Although Type I and Type III IFNs utilize distinct receptors for cell signaling, they activate similar pathways and thus exhibit in vitro activity against a similar array of viruses (e.g., hepatitis C virus [HCV], hepatitis B virus [HBV]) and regulation of host antiviral responses [14,15]. The receptor for Lambda comprises the IL-28 receptor Alfa (IL-28Rα) and IL-10 receptor beta (IL-10Rβ) chains, whereas all Type I interferons signal via a receptor complex of two unique IFN-α subunits, IFNAR1 and IFNAR2.

Key terms

Immunogenicity: The ability of a biotherapeutic to elicit an immune response.

Ligand-binding assay: Immunoassay-based laboratory technique commonly used to measure the concentration of a macromolecule in matrix. This technique is commonly used for pharmacokinetic assessments and frequently utilizes direct or indirect binding in sandwich and competition formats.

Lambda: 20 kDa interferon Lambda-1a linked to 20 kDa PEG.

Alfa: Pegasys, 20 kDa interferon Alfa-2a linked to a 40 kDa branched PEG.

Direct assay: Assay that uses different compounds for capture and detection of an analyte. In this work, biotinylated peginterferon (Lambda or Alfa) is complexed with an antibody directed against a PEG epitope that is then detected via a ruthenylated antibody against the constant domain of the PEG-bound antibody.

Selectivity: Ability of an analytical method to differentiate and quantify the analyte of interest in the presence of matrix constituents in the sample that may be expected to interfere in the assay.

Methods

Anti-PEG Ab detection methods

Bridge APAb assay

The APAb bridge assay utilizes Meso Scale Discovery (MSD) technology in which carbon electrodes integrated into the bottom of an assay plate excite a ruthenium label, emitting light at 620 nm which is then read by the MSD Sector Imager. Specifically, serum samples were diluted 1:2 in 1X PBS assay buffer with 1% BSA and 0.05% ProClin (no Tween-20) and are preincubated for 60 min at ambient room temperature (ART) followed by 18–24 h at 2–8°C with twice the volume of master mix (i.e., 25 µl pretreated sample and 50 µl master mix) containing 50 ng/ml biotinylated and 62.5 ng/ml ruthenylated PEG-IFN-λ (or 500 ng/ml biotinylated and 900 ng/ml ruthenylated PEG-IFN-α), allowing any APAb present in the sample to bind both labeled forms of the drug in a bridge complex. In the confirm tier, serum samples were diluted 1:2 in 1X PBS assay buffer as described above and in assay buffer containing 2 µg/ml 40 kDa branched PEG (or 2 µg/ml 40 kDa branched PEG in the alpha-based assay). A streptavidin coated MSD plate is blocked with assay buffer for 18–24 h at 2–8°C before adding 25 µl sample. Sample (with or without PEG) is incubated on the streptavidin plate for 2 h ± 10 min at ART, shaking at approximately 750 revolutions per minute (RPM). The drug-APAb complex is captured on the coated MSD microplates via the biotinylated portion of the complex. Upon addition of 2X MSD read buffer to the plate, the resulting light emission due to excitation of the ruthenium label is proportional to the amount of APAb present in the sample.

Negative control is prepared by pooling individual normal human serum screened in the PK and ADA assays to eliminate reactive individuals, those with endogenous interferon Lambda and/or pre-existing antibodies [16]. Positive control is prepared at the LPC (50 ng/ml) and HPC (500 ng/ml) by spiking the AGP4 IgM APAb (Sinica) into the NHS pool described above.

Direct APAb assay

The APAb direct assay also utilizes MSD technology. Biotin Alfa (1000 ng/ml) was added to streptavidin coated MSD plates and incubated for 60 ±5 minutes at ART. Serum sample or APAb, 6.3 IgG (Sinica) positive control, were diluted 1:100 in 1X PBS assay buffer with 1% BSA and 0.05% ProClin (no Tween-20), added to plates with immobilized Alfa and incubated for 1.5 h ±10 min at ART. Ruthenylated goat antihuman Ig was used to detect APAb bound to the plate. Assay sensitivity using the 6.3 PC was estimated to be approximately 25 µg/ml (concentration in

neat serum before the 1:100 minimum required dilution; MRD).

Anti-PEG Ab assay validation

The following assay validation parameters were examined: screening cut point, confirmatory (specificity) cut point, titer cut point, precision, assay range including sensitivity and hook effect, selectivity in normal and disease state serum, process temperature and freeze–thaw stability, robustness, drug tolerance and specificity.

Cut point

Data from all applicable cut-point runs, 16 plates run over 6 days by four analysts, were used to calculate the assay cut points, precision, and to determine assay acceptance criteria. Twenty (20) donor samples from the Hepatitis C (HCV) population and 34 donor samples from the normal human serum (NHS) populations were run in each assay as two sets of duplicates. In addition, two sets of the negative control (NC) and two sets of each of the positive controls (50 ng/ml low positive control, LPC; 500 ng/ml high positive control, HPC) were run as two sets of duplicates on each plate. For each donor and control sample, one set of duplicates was run with 40 kDa branched PEG at 1 µg/ml for determination of specificity cut point, and the other set of duplicates was run without PEG for determination of screen cut point. In all, each donor sample was assayed on four occasions, once by two analysts each, and twice by a third analyst. For the screening assay, the response values evaluated were the relative light units (RLU). For the confirmatory assay, the response values evaluated were the percent inhibitions.

The RLU for each sample w/o PEG was used to define an interim fixed assay cut point and a cut point factor to facilitate the implementation of a floating cut point consistent with currently published guidelines [17]. The interim fixed cut point represents the 95% upper confidence limit of the nonspecific background of the assay, corresponding to a 5% false positive rate when applied to study samples. Samples that were deemed to have pre-existing anti-PEG antibodies, based on signal inhibition in the confirmatory tier, were excluded from the cut point analyses. Additional analytical (outlier relative to other reported results for the same sample) and biological (outlier relative to the results of the other samples) outliers were identified and excluded from cut point calculations based on statistical justification.

The interim fixed cut point is based on log-transformed RLUs and is calculated as: Fixed CPI = EXP(MeanRLU + 1.645*Total SDRLU). 'MeanRLU' is the mean of the individual (log) RLU for the samples after outlier exclusion; '1.645' is the z-score from the normal distribution corresponding to the upper 5% tail area under the normal curve; 'Total SDRLU' is the estimate of the total standard deviation for the distribution of log RLU after outlier exclusion (based on an analysis of variance [ANOVA] model); and 'EXP' is the antilog expression of the fixed cut point calculation.

The multiplicative cut point factor (X) is calculated as: X = EXP(Fixed CPI - mean NCRLU). To account for plate-to-plate variability, this constant (X) is used to calculate the floating cut point on a plate-by-plate basis using the following equation: Floating Cut Point = mean NC (plate specific NC) * X.

Analyses were performed to verify that a common cut point could be applied to both populations (HCV and NHS). Evaluations included comparison of mean responses across populations using appropriate statistical procedures (e.g., ANOVA) to establish the degree of overlap between the populations.

The specificity cut point was determined using the mean response (RLU) from the individual donors (with and w/o excess PEG) and represents the 99% upper confidence limit of the nonspecific background of the assay in the presence of excess PEG corresponding to a 1% false positive rate when applied to study samples. The percent of signal inhibition for each of the individual donor samples is calculated as follows: Percent Inhibition = 100* (RLUw/o PEG - RLUw/ PEG) / RLUw/o PEG. For each donor, an estimate of the percent inhibition is calculated for each plate on which the sample is run. The specificity cut point is calculated using the following formula: Specificity Cut Point = Meanpct inhib + 2.33 * Total SDpct inhib. 'Meanpct inhib' is the mean percent inhibition for the donor population (after exclusion of outliers), '2.33' is the 'z-score' from the normal distribution corresponding to the upper 1% tail area under the normal curve; and 'Total SDpct inhib' is the estimate of the total standard deviation for the distribution of percent inhibition values after exclusion of outliers (based on an analysis of variance model). As with the screen cut point factor, a common cut point was applied for both subject populations.

Precision

Precision, consisting of coefficients of variation (%CV) for interanalyst, interday and interplate, and based on an analysis of variance model, was determined using all the HPC, LPC and NC from all applicable validation cut-point runs.

Drug tolerance

Samples containing PEGylated biotherapeutic, PEG or PEG analog can cause assay interference due to

competition for APAb between the PEG in the sample and PEGylated biotherapeutic in the assay. To assess how much free PEGylated biotherapeutic or free PEG was required to significantly reduce APAb detection in the assay, the LPC (50 ng/ml) and HPC (500 ng/ml) were spiked with multiple concentrations of free PEGylated biotherapeutic or free PEG (one, two, five, ten, 50 and 100 µg/ml) and were preincubated at room temperature for at least 1 h before analysis. The samples were assessed for positivity using the floating cut point for this experiment, 115 RLU. Drug tolerance was demonstrated at supraphysiological levels of Lambda, up to 2000 ng/ml at the LPC (50 ng/ml AGP4) and 10,000 ng/ml at the HPC (500 ng/ml AGP4). Drug tolerance was also demonstrated with free 40 kDa branched PEG up to 10,000 ng/ml at the HPC (500 ng/ml AGP4; data not shown).

Assay range (sensitivity & hook effect)

Data generated from sensitivity experiments are indicative of the positive control antibody population used and may not be reflective of antibodies that might be present in study subjects.

Relative assay sensitivity was determined by the lowest concentration of the APAb PC that produced a positive response in the screening and confirmatory assays. The PC was prepared at 2000 ng/ml in pooled normal human serum, was serially diluted twofold to 15.7 ng/ml in 100% pooled NHS, was diluted 1:2 in assay buffer (minimum required dilution) and analyzed with and without 1 µg/ml PEG (unconjugated 40KDa branched). Relative assay sensitivity was reported to be 15.7 ng/ml (Table 1).

Hook effect was examined similarly to assay sensitivity but at twofold dilutions ranging from 10,000 to 39 ng/ml. Although the signals begin to plateau at 625 ng/ml, the sample at 10,000 ng/ml still had a S/N ratio of 13.6 in the screen assay and 83% inhibition in the confirm assay (data not shown).

Specificity

APAb isotype

The ability of the APAb assay to measure both IgG and IgM APAb was evaluated by spiking the NC, LPC (50 ng/ml AGP4) and HPC (500 ng/ml AGP4) with three commercially available IgG monoclonal antibodies, 6.3, 3.3 and E11 (Sinica, Taiwan) and three custom IgG monoclonal antibodies at concentrations ranging from 37 ng/ml to 10 µg/ml. The signal to noise ratios were evaluated in the screen assay.

Per the Sinica specifications, AGP4 is a murine IgM monoclonal Ab that binds to the PEG backbone, PEG epitope ≥2000 Da; 6.3 is a second generation murine IgG1 monoclonal Ab that binds to the PEG backbone, PEG epitope approximately 750 Da; 3.3 is a first generation murine IgG1 monoclonal Ab that binds to the PEG backbone, PEG epitope ≥2000 Da; E11 is a murine IgG1 monoclonal Ab that binds to the PEG backbone, PEG epitope ≥2000 Da. Specifications of the custom Ab and further reagent characterizations of the commercial Ab will be published in a separate manuscript [18].

PEG size & conformation

PEG, size and conformation, cross-reactivity was evaluated by spiking the NC, LPC (50 ng/ml AGP4) and HPC (500 ng/ml AGP4) with a series of PEGylated therapeutics with variable PEG conjugations in addition to a series of free PEGs of differing size and conformation including: 20 kDa and 30kDa linear PEG-protein, 40 kDa branched PEG-protein (non-Pegasys),

Table 1. Relative assay sensitivity of the PEG-IFN-λ-based anti-polyethylene glycol antibody bridge assay using the AGP4 anti-polyethylene glycol IgM monoclonal antibody; cut point factor of 1.46 and confirmatory cut point of 34.8%†.

AGP4 concentration (ng/ml)	Screen			Confirm		
	Mean RLU	%CV	Ratio	Mean RLU	%CV	%Inhibition
2000	1723	4.6	20.3	426	1.7	75.3
1000	2060	4.2	24.2	430	0.3	79.1
500	1981	0.7	23.3	437	2.6	77.9
250	1648	1.7	19.4	399	0.0	75.8
125	1011	1.7	11.9	320	1.5	68.3
62.5	407	18.8	4.8	188	4.1	53.8
31.3	230	8.3	2.7	115	1.2	50.0
15.7	146	4.4	1.7	85	0.8	41.8

†Sensitivity in the PEG-IFN-α-based anti-PEG antibody assay was comparable.
CCP: Confirmatory cut point; CPF: Cut point factor; RLU: Relative light unit.

PEG-IFN-λ (20 kDa linear PEG), Pegasys (40 kDa branched PEG) unconjugated 20 kDa linear methoxy PEG (mPEG), 40 kDa branched mPEG and as a NC, non-PEGylated Lambda at concentrations ranging from 1 µg/ml to 100 µg/ml. The %inhibition were evaluated in the confirmatory (tier) assay.

Selectivity

Selectivity is the ability of an analytical method to differentiate and detect the analyte of interest in the presence of matrix constituents in the sample. Matrix components can result in interference and cross-reactivity, leading to false data. For example, matrix components, such as PEG or PEG analogs, can compete with PEG in the assay for APAb binding, resulting in false negative results. Conversely, matrix components that nonspecifically bind to PEG and bridge the assay can result in false positive results. To assess matrix effects, the HPC, LPC and NC were prepared using serum from 10 individual donors each from the NHS and HCV populations. Significant interference was observed in method development perhaps indicative of the presence of free PEG or structurally comparable analog. For the assay to be considered selective, 100% of the HPC spiked donor samples were required to test positive but only ≥70% of the LPC spike donor samples were required to test positive. No limit was placed on the number of blank (NC) donor samples that could test positive presumably due to pre-existing APAb. Data generated from the selectivity experiments reflect the affinity and avidity of the AGP4 (and 6.3, data not shown) positive control antibody population and may not be reflective of antibodies that might be present in subject samples.

Clinical studies

Samples used for Lambda/Alpha protein domain cross-reactivity testing, described below, were from the AI452–017 double blind, randomized, multicenter trial in treatment of naive chronic HCV GT2,3 infected subjects comparing the safety and efficacy of PEG-IFN-λ/Ribavirin (RBV) or PEG-IFN-λ/RBV/Daclatasvir (DCV) with Alfa (Pegasys®)/RBV co-administration. Duration of treatment in this study for PEG-IFN-λ/RBV and Alfa/RBV was 24 weeks.

Samples used for APAb testing were from the AI452–005 double blind, randomized, multicenter trial in treatment of chronic hepatitis B infected HBeAg+ subjects who had not had prior exposure to interferon. The objective of this trial was to compare the safety and efficacy of 48 weeks treatment with PEG-IFN-λ or PEG-IFN-α monotherapy.

Cryoglobulinemia, lymphoma and preexisting autoimmune diseases were exclusionary criteria in these clinical studies. All testing described herein is in compliance with clinical study patient consent.

Key term

Cross-reactivity: When an antibody raised against a certain compound binds to a compound of high structural similarity. For example: various PEG sizes and conformations or proteins within the interferon super family.

Lambda/Alfa cross-reactivity

One hundred nineteen (119) subjects from the Lambda treatment group (Study AI452–017), that tested positive for Ab to Lambda in the Lambda-based AIAb bridge assay (cut point factor, CPF 1.2; confirmatory cut point, CCP 17.4%), were tested for cross-reactivity to Alfa in the Alfa-based AIAb bridge assay (CPF 1.22, CCP 27.2%). The subset subject population selected for cross-reactivity testing included all subjects in a pivotal PIII clinical study from the top three enrolled countries. Anti-Lambda Ab titers for this 119 subject subset ranged from <5 to 78,125.

AIAb & APAb clinical study (AI452–005) sample analysis

One-hundred seventy-three (173) drug naive subjects from the AI452–005 study were treated with either PEG-IFN-λ or PEG-IFN-α once weekly for up to 48 weeks. Blood samples for analysis of antidrug antibodies, AIAb and APAb, in serum were generally obtained at Day 1 (predose), and weeks two, four, 12, 16, 24, 40 and 48, and during post treatment period at weeks 60, 72, 96, 120, 144, 168 and 192.

Three validated bridging electrochemiluminescent (ECL) methods were used for the detection of antibodies to: 1. the Lambda protein domain, 2. the Alfa protein domains and to 3. PEG (Alfa treated subjects). A fourth qualified method was used to detect APAb in Lambda treated subject samples. As discussed in the APAb detection strategy above and in the cross-reactivity results section below, given the lack of cross-reactivity of Ab to the protein domains of the two comparator compounds, the conventional ADA bridge assays were optimized for the detection of APAb. The PEG-IFN-λ-based assay was used to test samples from subjects treated with PEG-IFN-α and the PEG-IFN-α-based assay was used to test samples from subjects treated with PEG-IFN-λ. Detergent was removed from the assay buffers and wash buffers due to the cross-reactivity of Tween (and all other effective detergents tested) with the APAb in the assay. Additionally, the titers of AIAb were so high that the reactivity of Ab to the protein domains obscured the APAb results. Thus, to eliminate this interference the PEG-IFN-α-based assay

was used to test for APAb in samples from subjects treated with PEG-IFN-λ and vice versa. The PEG-IFN-α-based assay validation was discontinued due to program termination and not due to the inability to meet validation criteria. A fifth direct assay was used to detect APAb (IgG) that may have not been detected by the bridge assay. A sixth validated cell-based neutralizing antibody assay was used to detect neutralizing antibodies in PEG-IFN-λ treated subjects and will be described in an independent manuscript.

Determination of immunogenicity incidence

Subjects were considered ADA (AIAb or APAb) positive if there was seroconversion from the baseline time point to a specific and measurable ADA titer at any time point throughout the sampling period. For a subject to be considered AIAb positive, the subject was required to have at least two consecutive ADA positive time points approximately 12-weeks apart. This criteria was imparted due to the frequency of AIAb with the objective of increasing the probability of correlation between AIAb events and loss of efficacy. As all subjects with AIAb had at least two consecutive AIAb, there was no impact on the incidence using this design. Due to the infrequency of APAb, this criterion was not employed for the APAb end point. For a subject to be considered APAb positive, the subject was required to have at least 1 APAb positive result at any time point. For subjects with pre-existing antibodies at baseline, measurable increases in titer (defined as ≥5-fold) were reported. Subjects with pre-existing antibodies at baseline who did not have at least a fivefold increase in titer at a post-baseline visit were considered to have a static response. Subjects with at least two consecutive ADA positive time points, per seroconversion or boosting criteria, were considered to have a persistent antibody response.

Results & discussion

Lambda/Alfa cross-reactivity

One hundred nineteen (119) subjects from the AI452–017 study that were positive for Lambda ADA were tested for cross-reactivity to Alfa. Both the Lambda and Alfa ADA assays are conventional ECL-based bridge formats. The Lambda ADA assay has a cut point factor (CPF), used to determine putative positive samples in the screen tier, of 1.20 and a confirmatory cut point (CCP), used to verify ADA positivity, of 17.4% inhibition. Similarly, the Alfa ADA assay has a CPF of 1.22 and a CCP of 27.2% inhibition. Both assays have relative assay sensitivity of approximately 10 ng/ml using specific positive controls derived from hyper-immunized cynomolgus monkeys. Only 1/119 (<1%) subjects was positive for ADA to both Lambda and Alfa. This subject had pre-existing anti-Lambda and anti-Alfa Ab and no boosting was observed for either. In other terms, there was no treatment emergent or treatment induced Ab pertaining to this subject. Titers for this subject were substantially higher for anti-Lambda Ab, approximately 200,000 RLU than anti-Alfa Ab, approximately 200 RLU (Table 2). Anti-Lambda Ab titers in the 119 subject subset ranged from <5 to 78,125. All 119 subjects had persistent anti-Lambda Ab and only the single subject noted above had anti-Alfa Ab. Thus the subject population was deemed sufficient to demonstrate lack of cross-reactivity of anti-Lambda Ab to Alfa. These results are as expected based on what is known about these two distinct IFNs which have unique protein sequences and conformations and bind distinct targets.

Anti-PEG Ab bioanalytical strategy

Given the lack of cross-reactivity of anti-Lambda Ab to Alfa and anti-Alfa Ab to Lambda, the optimized PEG-IFN-λ bridge assay was used to test samples from subjects treated with PEG-IFN-α and the PEG-IFN-α bridge assay was used to test samples from subjects treated with PEG-IFN-λ. Each assay was deemed suitable for detecting APAb without the interference from anti-protein Ab. Subsequent tier analysis included testing in a direct assay for improved detection of IgG APAb.

Anti-PEG Ab assay validation

Cut point

Eight out of the 54 (15%) commercially procured donor samples (six NHS; two HCV), where the overall percent inhibition was >30%, were considered to have pre-existing antibodies and were excluded from cut point determination.

In the screen assay, the populations had overlapping ranges: NHS, 60–132 and HCV, 60–115, excluding eight donors attributed to have pre-existing APAb (biological outliers), two donors attributed to have pre-existing AIAb and one donor attributed as an analytical outlier. It should be noted that other studies have shown pre-existing AIAb rates to be higher than what was observed in this testing [16]. Sample means were 79 (NHS) and 84 (HCV), and standard deviations were similar, 18 (NHS) and 24 (HCV). An ANOVA to evaluate mean differences in RLUs showed there to be no significant difference in means across populations ($p = 0.55$). These descriptive and inferential assessments suggested that it was appropriate to combine RLUs across populations and report one cut point and cut point factor for the NHS and HCV populations. Calculation of the fixed cut point and cut point factor for the combined distribution was performed based on log-transformed data. The cut point factor was

Table 2. Antibodies to the Lambda protein domain do not cross-react with the Alfa protein domain†.

Subject	Treatment	Time	PEG-Lambda screen RLU	PEG-Alpha screen RLU	ADA result
1	PEG-IFN-λ	Day 1 (Baseline)	302960	207	Positive for ADA to PEG-Lambda and PEG-Alpha
		Week 2	271260	205	
		Week 4	236780	189	
		Week 12 (EOT)	185646	206	
2–119	PEG-IFN-λ	Day 1 (Baseline)	100–302,960	44–74	At least 1 time point positive for ADA to PEG-Lambda, negative for ADA to PEG-Alpha
		Week 2			
		Week 4			
		Week 12 or 24 (EOT)			
Mean NC			63	53	–
CPF			1.2	1.22	–
CCP			17.4	27.2	–

†Only 1 out of 119 subjects positive for anti-Lambda antibody also had anti-Alfa antibody. In this case, the antibodies to both Lambda and Alfa were pre-existing (positive at baseline) and there was no treatment related boosting. Note: These data are not a good indicator of anti-PEG antibody due to the levels of Tween-20 in the assay.
ADA: Anti-drug antibody; CCP: Confirmatory cut point; CPF: Cut point factor; EOT: End of treatment; NC: Negative control; RLU: Relative light unit.

determined to be 1.46 for the combined set of donor sample results. Since the factor is multiplicative, the plate cut point is calculated by multiplying the cut point factor by the NC RLU mean for the plate. There was strong overlap between the donor samples and NC RLUs across all plates indicating the suitability of the NC for use in a floating cut point paradigm.

In the specificity assay, the populations again had overlapping ranges: NHS, -14 to 28% and HCV, -11 to 25% excluding eight biological outliers (pre-existing APAb) and one analytical outlier. Sample means were 6% (NHS) and 4% (HCV), and standard deviations were similar (10–11). No significant difference in means between the NHS and HCV populations was observed ($p = 0.46$), suggesting that it was appropriate to combine specificity assay results across populations and report one specificity cut point. The cut point was determined to be 34.8% using the 99% confidence level (44.4% at the 99.9% level).

Sensitivity

Data generated from sensitivity experiments are indicative of the positive control antibody population used and may not be reflective of antibodies that might be present in study subjects. With the specific objective of trying to develop an assay that would be sufficient to detect a diverse set of APAb, representative of a human population, numerous APAb were evaluated including: AGP3, AGP4, 3.3, 6.3, E11 (Sinica), B47 (Epitomics), and three internally generated custom APAb [18]. Per the Sinica specifications, AGP3 and AGP4 are murine IgM monoclonal Ab; 3.3, 6.3 and E11 are murine IgG1 monoclonal Ab; all are purported to bind to the PEG backbone. B47 (Epitomics) is a rabbit IgG monoclonal Ab that reportedly binds to the terminal methoxy group of the PEG. Specifications of the custom Ab and further reagent characterizations of the commercial Ab will be published in a separate manuscript [18].

Relative assay sensitivity was determined by the lowest concentration of the APAb PC that produced a positive response in the screening and confirmatory assays. The AGP4 PC was used to determine sensitivity in the bridge assay and was approximated to be 15.7 ng/ml in 100% pooled NHS (Table 1). The 6.3 PC was used to determine sensitivity in the direct assay and was approximated to be 25 µg/ml in 100% pooled NHS (data not shown). Reactivity of the additional APAb is discussed in the specificity section below.

Specificity

APAb isotype detection (cross-reactivity) was evaluated in the bridge assays by spiking the NC, LPC (50 ng/ml AGP4) and HPC (500 ng/ml AGP4) in 100% NHS with three commercially available IgG monoclonal antibodies, 3.3, 6.3 and E11 (Sinica, Taiwan), three custom IgG monoclonal antibodies (data not shown) and two polyclonal antibodies raised against either PEG-IFN-λ or PEG-IFN-α at concentrations ranging from 37 ng/ml to 10 µg/ml. Detection of IgM and IgG APAb: AGP4, 6.3, 3.3, E11 (Sinica) and the antiPEG-IFN-α PC in the PEG-IFN-λ-based bridge APAb assay are shown in Table 3. All Ab were detectable in a concentration dependent manner.

The 6.3 IgG APAb at 500 ng/ml had a S/N ratio of 7.5 when spiked with 50 ng/ml AGP4 as compared with a 2.8 ratio of 50 ng/ml AGP4 alone. Similarly, the E11 IgG APAb at 500 ng/ml had a ratio (S/N) of 8.0 when spiked with 50 ng/ml AGP4 as compared with a 2.8 ratio of 50 ng/ml AGP4 alone. The 3.3 IgG APAb was not detectable at 500 ng/ml but was detectable at 10,000 ng/ml. The anti-Alfa PC was modestly detected even at relatively high levels. Binding (S/N ratio) of the three custom APAb was similar to that of 3.3. Reactivity of all APAb (AGP4, 6.3, 3.3 and E11) was comparable in the Alfa-based APAb assay. The anti-Alfa and anti-Lambda PC were raised against PEGylated therapeutic (Lambda or Alfa) in hyper immunized cynomolgus monkeys and were affinity purified against PEGylated therapeutic so it is possible that a portion of the polyclonal Ab population is directed against PEG although from these data it does not appear to be a predominant portion.

PEG, size and conformation, cross-reactivity was evaluated by spiking the NC, LPC (50 ng/ml AGP4) and HPC (500 ng/ml AGP4) with a series of PEGylated therapeutics with variable PEG conjugations in addition to a series of free PEGs of differing size and conformation including: unconjugated 20 kDa linear methoxy PEG (mPEG), unconjugated 40 kDa branched mPEG, PEG-Lambda (20 kDa linear PEG), Pegasys (40 kDa branched PEG), and as a negative control, non-PEGylated Lambda (data not shown) at concentrations ranging from 1 µg/ml to 100 µg/ml. PEG cross-reactivity in the APAb Lambda-based bridge assay at 5 µg/ml 20 kDa and 30 kDa linear and 40 kDa branched PEG, conjugated and unconjugated to protein is shown in Table 4. The specificity cut point was 34.8% using the 99% confidence interval. All PEG variations were shown to compete for APAb binding and to inhibit signal by greater than the 34.8% specificity cut point. The nonpegylated interferon did not sufficiently immunodeplete the sample. Moreover, when excess PEG was spiked into the confirmatory tier with AIAb, there was no immunodepletion indicating that the immunodepletion observed with the APAb was due to soluble PEG competing for APAb binding and not PEG-mediated precipitation (Supplemental Table 1S). The Alfa-based APAb assay was also shown to detect APAb reactive with the PEG sizes and conformations discussed above.

Table 3. Detection of IgM and IgG APAb: AGP4, 6.3, 3.3, E11 (Sinica) and the anti- PEG-IFN-α positive control (anti-Alfa) in the PEG-IFN-λ-based bridge anti-polyethylene glycol antibody assay[†].

AGP4 concentration (ng/ml)	Cross-reactive Ab	Mean RLU	%CV	S/N ratio
50	NA	228	4	2.8
	6.3(10,000 ng/ml)	2050	4	25.3
	6.3(500 ng/ml)	611	4	7.5
	3.3(10,000 ng/ml)	501	4	6.2
	3.3(500 ng/ml)	231	2	2.9
	E11(10,000 ng/ml)	1469	12	18.1
	E11(500 ng/ml)	651	4	8.0
	Anti-Alfa(10,000 ng/ml)	298	1	3.7
	Anti-Alfa(500 ng/ml)	256	6	3.2
500	NA	1034	13	12.8
	6.3(10,000 ng/ml)	2855	1	35.2
	6.3(500 ng/ml)	1585	1	19.6
	3.3(10,000 ng/ml)	885	28	10.9
	3.3(500 ng/ml)	765	5	9.4
	E11(10,000 ng/ml)	2200	4	27.2
	E11(500 ng/ml)	1494	1	18.4
	Anti-Alfa(10,000 ng/ml)	1012	40	12.5
	Anti-Alfa(500 ng/ml)	1243	8	15.3

[†]The NC had a mean RLU of 81.
Ab: Antibody; Anti-Alfa: An antibody with specificity to IFN-α; NA: Not applicable; NC: Negative control; RLU: Relative light unit.

Table 4. Polyethylene glycol cross-reactivity in the PEG-IFN-λ-based bridge anti-polyethylene glycol antibody assay: 5 μg/ml 20 kDa and 30 kDa linear and 40 kDa branched, conjugated and unconjugated to protein shown†.

Screen			
Sample ID	**Mean RLU**	**%CV**	**S/N ratio**
NC	81	6	NA
LPC (Conc.:50ng/ml)	377	0	4.7
HPC (Conc.:500ng/ml)	1864	1	23.0
Specificity			
Sample ID	**Mean RLU**	**%CV**	**%Inhibition**
LPC w/ 20K linear PEG	197	2	47.7
HPC w/ 20K linear PEG	747	3	59.9
LPC w/ 40K branched PEG	181	0	51.9
HPC w/ 40K branched PEG	181	2	90.3
LPC w/ 20K linear PEG-protein	67	2	82.2
HPC w/ 20K linear PEG-protein	188	3	89.9
LPC w/ 30K linear PEG-protein	110	1	70.8
HPC w/ 30K linear PEG-protein	183	2	90.2
LPC w/ 40K branched PEG-protein	80	0	78.8
HPC w/ 40K branched PEG-protein	96	2	94.8

†Cross-reactivity in the PEG-IFN-α-based APAb assay was comparable. The specificity cut point was 34.8% using the 99% confidence interval.
HPC: High positive control; LPC: Low positive control; NC: Negative control; RLU: Relative light unit.

Due to the structural similarity of PEG and many detergents, numerous detergents were evaluated during method development for interference with APAb binding including: Tween 20, Triton X-100, Brij 35 and NP-40. All were shown to significantly impede APAb binding in the assay. Detergents with structural uniqueness to PEG were also tested including CHAPS and OTG, but were not shown to improve signal to noise and thus were not employed (data not shown).

Selectivity

Matrix components can result in interference and/or cross-reactivity, leading to false data. For example, matrix components, such as PEG or PEG analogs, can compete with PEG in the assay for APAb binding, resulting in an underestimation of APAb events (false negative results). Conversely, multimeric matrix components other than APAb that bind to PEG and bridge the assay can result in an overestimation of APAb events (false positive results). To assess matrix effects (selectivity), the HPC, LPC and Blank (NC) were prepared using serum from 10 individual donors each from the NHS and HCV populations. Three (3) out of 10 (30%) HCV donors w/o exogenously spiked APAb (blank) were positive for pre-existing APAb. All had low titer (≤151 RLU; CP 124 RLU). One (1) out of 10 (10%) HCV donors w/o exogenously spiked APAb (blank) was positive for pre-existing AIAb. The subject with pre-existing AIAb had a relatively high titer (6116 RLU) as compared with the subjects with pre-existing APAb (≤151 RLU). Ten (10) out of 10 (100%) HCV donors spiked at LPC and HPC with AGP4 APAb were positive for APAb, however, there was a relatively high degree of variability as shown in Figure 1. Two (2) out of 10 (20%) NHS donors w/o exogenously spiked APAb (blank) were positive for pre-existing APAb (low titer, ≤154 RLU). Eight (8) out of 10 (80%) NHS donors spiked at LPC and 10 out of 10 (100%) spiked at the HPC with AGP4 APAb were positive for APAb. Similarly to the HCV population, a relatively high degree of variability amongst the donors spiked with APAb was observed (data not shown). It is uncertain whether the variability in spike recovery is due to endogenous PEG or PEG analogs, the low affinity of APAb or another attributable but unknown cause. It has been postulated that the APAb, specifically IgM, binds not only to PEG but to polysaccharides and other antigens that have long, less digestive, repeated structures. This could be one of the reasons that we observed variable spike recovery in the APAb spiked selectivity samples. Conversely, the pre-existing APAb could actually be heterophilic and cross-reactive but not due to PEG exposure. These experiments will be included in our follow-up research.

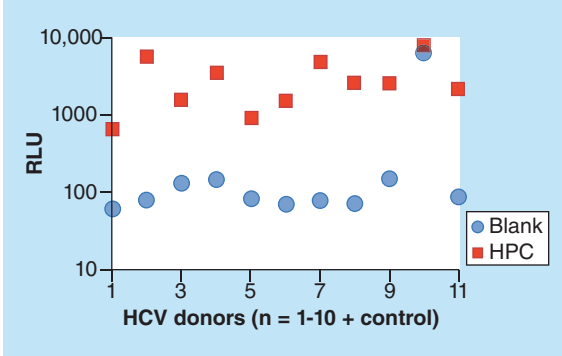

Figure 1. Anti-polyethylene glycol antibodies assay selectivity using AGP4 in ten hepatitis C virus donor's serum at blank and high positive control. Control in pooled normal human serum (healthy). Cut point at 124 RLU. 20% HCV donors were positive for pre-existing anti-PEG antibodies. 100% donors spiked with AGP4 were positive for anti-PEG antibodies, although interdonor variability was relatively high.
HCV: Hepatitis C virus; HPC: High positive control; RLU: Relative light unit.

Clinical results

Of 80 subjects treated with Lambda in the AI452–005 clinical study, 79 were evaluated for AIAb, and among these subjects, 8 of 79 (10.1%) had pre-existing AIAb detectable at baseline. Of the 79 subjects with evaluable ADA data, 47 (59.5%) seroconverted or had a boosted response on treatment. Forty-five (45) subjects seroconverted; and of the 8 subjects with pre-existing AIAb 2 (25%) had a boosted response. The first subject to have positive AIAb samples was at Week 2 while for most ADA positive subjects the onset was around Week 12. For subjects that developed AIAb, the response persisted and the number of positive subjects continued to rise through the end of treatment. There is not sufficient recovery period data at this time to assess reversibility; long term follow-up data collection is in progress.

Of the 80 subjects randomized to the Lambda group (AI452–005), 32 were evaluated for APAb, and among these subjects, 2 of 32 (6.3%) had pre-existing APAb detectable at baseline. Eight of the 32 (25%) subjects had at least one positive sample for APAb, of which 2 had pre-existing antibodies and did not have a boosted response, 6 had transient responses, that is, did not have two consecutive timepoints positive for APAb, and 2 had persistent responses. All subjects who had at least one positive sample for APAb also had AIAb. One subject had APAb detected in the APAb direct assay that were not detected in the APAb bridge assay at 2, 12 and 16 weeks. However, both the bridge and direct APAb assays detected APAb in this subject's week 4 sample and AIAb were detected for this subject at weeks 2, 12, 16, 24, 40, 48 and 72. In general, AIAb were more frequent, more persistent and were of higher titer than APAb (Figure 2). A representative time course of AIAb and APAb is shown in Table 5. While only 4 out of 54 (7.4%) subjects treated with either Lambda or Alfa had persistent APAb, we have included an example time course in Table 6. The clinical time course of APAb and AIAb provide some evidence that epitope spreading could be occurring. APAb are generally earlier onset, transient and are followed by AIAb. However, 72% of subjects had AIAb (Lambda) and only 25% had APAb so while this is possible, more data is needed to fully understand any causal relationship.

Of 83 subjects treated with Alfa (AI452–005), 82 were evaluated for AIAb. One (1) out of the 82 subjects (1.2%) had pre-existing AIAb, which did not boost with treatment. Twenty-seven (27) of the 82 subjects (32.9%) seroconverted while on treatment.

Of the 83 subjects treated with Alfa (AI452–005), 22 were evaluated for APAb. Among the 22 subjects, 2 (9.1%) had pre-existing APAb detectable at baseline. Six (6) of the 22 (27%) subjects had at least one positive APAb sample, of which 2 had pre-existing APAb, 4 had transient samples and 2 had persistent responses. Thus, 2 of 22 (9.1%) subjects tested had a persistent APAb response. All subjects that tested positive for APAb also tested positive for AIAb. One subject had APAb detected in the APAb direct assay that were not detected in the APAb bridge assay at 40, 48 and 72 weeks. However, both the bridge and direct APAb assays detected APAb in this subject's Day 1 and week 2, 4, 12, and 24 sample and AIAb were detected for this subject at weeks 12, 16, 24, 40, 48 and 72. Similar to the Lambda arm, in the Alfa arm, AIAb were more frequent, persistent and higher titer than APAb.

The number of subjects in the treated populations with any APAb, regardless of whether the response was persistent, was small in comparison to subjects with the AIAb responses. Not only are the number and percentages of subjects with AIAb much greater than those with APAb, but the magnitude of responses is much greater in these subjects as well. In examining the time course of APAb versus AIAb, the onset of AIAb is later and more consistent than the APAb response (Table 5). Because of the low frequency and magnitude of APAb responses, these events would not be expected to have clinical impact with regard to effects on PK, efficacy or safety. There were no reported hypersensitivity events related to PEG-IFN-λ in the Phase II studies reported here. Phase III results are pending at the time of this manuscript.

For subjects treated with pegylated IFNs, who had the presence of APAb at baseline, their APAb responses did not boost upon treatment. This suggests that pre-existing Abs directed against PEG, which possibly

originated from previous PEG exposure, such as from consumer products, does not boost in response to treatment with pegylated IFN described herein. It is unknown if the few subjects who had the persistent APAb responses will continue to be cross-reactive and have responses against PEG from other sources. The persistent APAb responses were of lower magnitude in comparison to the ADA directed against the IFN portion of the molecules, suggesting that the responses are of low level and may have little to no impact clinically for the molecules.

Conclusion

A surrogate peglyated protein can serve as a basis for an APAb assay

A multipronged approach was used to detect APAb in a clinical program comparing a novel Lambda to Alfa. First, human serum samples from each treatment group (Lambda or Alfa) were tested for cross-reactivity to the comparator protein using the conventional Lambda or Alfa-based bridging assays. For example, samples from Lambda treated subjects, known to be positive for ADA to Lambda were tested in the Alpha-based assay. Since there was no measurable cross-reactivity of Lambda specific ADA (PC and subject samples) to the Alfa protein domain and vice versa, the conventional ADA bridge assays were optimized for the detection of APAb. The PEG-IFN-λ-based bridge assay, optimized to detect APAb, was used to test samples from subjects treated with PEG-IFN-α for APAb and the PEG-IFN-α-based bridge assay was used to test samples from subjects treated with PEG-IFN-λ for APAb. Any reactivity in the Alfa-based APAb assay from Lambda treated subjects and vice versa was deemed likely to be from APAb as it is the epitope both treatment groups have in common.

The bridge assay detects IgM & IgG APAb reactive to common PEG derivatives at sufficient sensitivity

Each assay was deemed suitable for detecting APAb reactive with multiple PEG sizes and conformations (20, 30 or 40 kDa linear and 40 kDa branched) both conjugated to protein and unconjugated. Although the bridge assay was able to measure both IgM and select IgG antibodies, the bridge format has been theorized to be preferential for IgM detection. As we did not have conclusive evidence to the contrary, a direct assay format was sequentially developed and employed. Samples from all time points for subjects that tested positive at any one time point for APAb in the more sensitive bridge assay were then analyzed in the direct assay. The intention of this serial analysis was to use the more sensitive bridge assay to detect early IgM APAb and then use the less sensitive direct assay to measure subsequent IgG APAb. Note

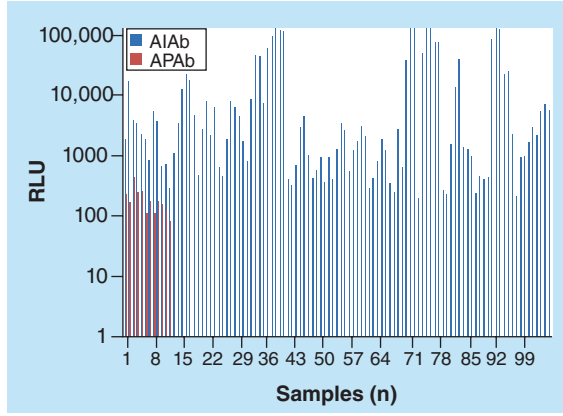

Figure 2. Anti-interferon antibody are more frequent, persistent and have higher titer than anti-PEG antibody. n = 105 samples.
AIAb: Anti-interferon antibody; APAb: Anti-PEG antibody; RLU: Relative light unit.

that this sensitivity sequence is applicable to detection in human serum where excess human immunoglobulin that passively absorbs to the plate binds to the detection Ab and causes high background noise in the direct assay. As the bridge assays were theoretically compromised for detecting IgG APAb, the entire sample time course was tested in the direct assay for subjects with even a single positive result in the bridge assay. Only one subject had APAb in the direct assay that were not also detected in the bridge assay. More data is needed to determine the necessity for testing in both formats.

The clinical data support the concept that while some subjects treated with PEGylated therapeutics may exhibit APAb responses, these responses are typically observed as low level response, both frequency and titer, in the population, and most likely do not contribute to clinical manifestations during treatment with either of these IFNs or to other PEGylated therapeutic proteins.

Future perspective

Several PEGylated products have been approved by health authorities and have been on the market for sufficient periods of time with little evidence of chronicity of anti PEG immunogenic response and minimal manifestations that would link such an immune response to the product's efficacy or safety performance. However, this has not mitigated concerns and expectations faced by drug developers on the need for

Key term

Bridging assay: Assay that uses the same unique reagent for both capture and detection of an analyte. In this work, biotinylated peginterferon (Lambda or Alfa) and ruthenylated peginterferon (Lambda or Alfa) bridged (or complexed) by antibody directed against a shared PEG or protein epitope.

Table 5. Representative anti-interferon antibody and anti-polyethylene glycol antibody time course for a subject that tests positive for anti-polyethylene glycol antibody†.

Time point	AIAb (bridge)	APAb (bridge)	APAb (direct)
Day 1	-	-	-
Week 2	-	+	-
Week 4	-	-	-
Week 12	-	-	-
Week 16	+	-	-
Week 24	+	-	-
Week 40	+	-	-
Week 48 (EOT)	+	-	-
Week 72 (EOF)	+	-	-

†All subjects that test positive for APAb also test positive for AIAb albeit the timing is not usually coincident, as shown below. APAb tend to be earlier onset and transient while AIAb tend to be later onset (relatively) and persistent.
AIAb: Anti-interferon antibodies; APAb: Anti-PEG antibodies; EOF: End of follow-up (recovery period); EOT: End of treatment.

Table 6. Example anti-interferon antibody and anti-polyethylene glycol antibody time course for a subject that tests persistently positive for anti-polyethylene glycol antibody. Only four out of 54 (7.4%) subjects treated with either Lambda or Alfa had persistent anti-polyethylene glycol antibody†.

Time point	AIAb (bridge)	APAb (bridge)	APAb (direct)
Day 1	-	+	+
Week 2	-	+	+
Week 4	-	+	+
Week 12	+	+	+
Week 16	+	-	+
Week 24	+	+	+
Week 40	+	-	+
Week 48 (EOT)	+	-	+
Week 72 (EOF)	+	-	+

†All subjects that test positive for APAb also test positive for AIAb albeit the timing is not usually coincident, as shown below. APAb tend to be earlier onset and transient while AIAb tend to be later onset (relatively) and persistent.
AIAb: Anti-interferon antibodies; APAb: Anti-PEG antibodies; EOF: End of follow-up (recovery period); EOT: End of treatment.

robust and reliable methods to demonstrate evidence (or lack thereof) of anti PEG immunogenicity.

Unlike immune responses to proteins that can be driven to affinity maturation following chronic exposure, the immunogenicity to PEG can span a wide spectrum ranging from low affinity interactions to moderately high. Furthermore the responses can be skewed depending on whether there were pre-existing antibodies that naturally arise following long term exposure to PEG in nutritional and cosmetic products. In addition, the oversimplification of anti-PEG antibodies being either anti backbone or anti methyl cap or anti linker may compromise our understanding of the effect of length and branching structure of the PEG utilized in an assay to identify anti PEG antibodies. Clearly the analytical challenge is to develop assays designed not just for any IgG response to PEG but rather reflecting the innate diversity of anti PEG antibodies in human subjects – capable of detecting low affinity antibodies, pre existing antibodies and boosted responses elicited by the PEG conjugate specific to the therapeutic product. An added challenge is detecting antibodies that might differentially bind to the backbone subunit based on PEG conformation (size and structure). Each of these categories of IgG responses is likely to have different analytical outcomes in any one assay. Unlike the conventional approaches to developing materials and methods for a typical anti protein therapeutic IgG assay, the antibody response to PEG poses new questions and compels us to think about a need for wider array of well characterized reagents whose analytical behavior in an immunogenicity assay should be reflecting those found

in the human subjects. The methods and reagents need to be shared and standardized across different PEGylated platforms amenable for comparative and cooperative research before one can confidently rule in or out the role of PEG in immune mediated aberrations on the pharmacological activity of the therapeutic.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/BIO.15.36

Acknowledgement

The authors acknowledge the following BMS team members for their contributions to this manuscript H Brett-Smith, C Jurkowski, D Xu, S Portsmouth, M Wind-Rotolo, C Impellizzeri, L Critelli, S Neumann, R Pillutla, J Haulenbeek, L Williams, J Duo, H Vasudevan, P Kadiyala, D Stocker, B Stouffer, partners at Tandem Laboratories, Trenton NJ and Wuxi AppTech, Shanghai China. The authors also acknowledge the following external parties for their review of this manuscript: B Gorovits, A Rosenberg, L Salazar-Fontanaand M Subramanyam.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Extensive use of polyethylene glycol (PEG) in consumer products could becausative for increased prevalence of anti-PEG antibodies (APAb) in humans
- APAb methods are challenging due to the repeating motif structure of PEG, the relatively low affinity of anti-PEG Abs, the comparability of PEG structure to detergents and the challenges of producing relevant positive controls.
  - APAb assays should be able to detect IgG and IgM APAb.
  - APAb assays should be able to detect APAb that cross-react with a wide range of common PEG sizes and derivatives.
  - A surrogate pegylated protein that is structurally distinct from your therapeutic protein can serve as a basis for an APAb assay.
- Of 54 subjects evaluated for APAb (AI452-005), 6% of PEG-IFN-λ and 9% of PEG-IFN-α subjects had persistent APAb. Fourteen (14) of 54 (26%) subjects had at least one positive sample for APAb. Of 161 subjects evaluated for AIAb, 60% of PEG-IFN-λ and 33% of PEG-IFN-α subjects had persistent AIAb.
- Of the 80 subjects randomized to the PEG-IFN-λ treatmentgroup (AI452-005), 2 of 32 (6%) had pre-existing APAb, 8 of 79 (10%) had pre-existing AIAb. Boosting events for AIAb or APAb were uncommon.
- Generally, AIAb were significantly more frequent, persistent and higher titer than APAb. Clinical impact will be discussed in a separate manuscript.

References

Papers of special note have been highlighted as:
• of interest

1 Sailstad JM, Amaravadi L, Clements-Egan A *et al.* A white paper-consensus and recommendations of a global harmonization team on assessing the impact of immunogenicity on pharmacokinetic measurements. *AAPS J.* 16(3), 488–498 (2014).

2 Parenky A, Myler H, Amaravadi L *et al.* New FDA draft guidance on immunogenicity. *AAPS J.* 16(3), 499–503 (2014).

• **Industry-FDA collaborative manuscript summarizing the key aspects of the 2014 US FDA 'Guidance for Industry: immunogenicity assessment for therapeutic protein products.'**

3 Stevenson L, Amaravadi L, Myler H *et al.* LM by LBA and immunogenicity. *Bioanalysis* 6(24), 3355–3368 (2014).

4 US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research. Guidance for industry: immunogenicity assessment for therapeutic protein products. FDA, Rockville, MD, USA (2014). www.fda.gov

• **US FDA Guidance describing the bioanalytical requirements for anti-drug Ab testing.**

5 US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research. Draft guidance for industry: assay development for immunogenicity testing of therapeutic proteins. FDA, Rockville, MD, USA (2009). www.fda.gov

• **FDA Guidance describing the requirements for anti-PEG Ab testing.**

6 Garay RP, El-Gewely R, Armstrong JK *et al.* Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opin. Drug Del.* 9(11), 1319–1323 (2012).

7 Schellekens H, Hennink WE, Brinks V. The immunogenicity of polyethylene glycol: facts and fiction. *Pharm. Res.* 30, 1729–1734 (2013).

8 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. Meth.* 64(3), 238–245 (2011).

9 Armstrong JK, Hempel G, Koling S *et al.* Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* 110, 103–111 (2007).

10 Ganson NJ, Kelly SJ, Scarlett E *et al.* Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a Phase I trial of subcutaneous PEGylated urate oxidase. *Arthritis Res. Ther.* 8(R12), 1–10 (2006).

11 Hershfield MS, Ganson NJ, Kelly SJ *et al.* Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. *Arthritis Res. Ther.* 16(R63), 2–11 (2014).

12 Sheppard P, Kindsvogel W, Xu W *et al.* IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat. Immunol.* 4(1), 63–68 (2003).

13 Kotenko SV, Gallagher G, Baurin VV *et al.* IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat. Immunol.* 4(1), 69–77 (2003).

14 Robek MD, Boyd BS, Chisari FV. Lambda interferon inhibits hepatitis B and C virus replication. *J. Virol.* 79(6), 3851–3854. (2005).

15 Doyle SE, Schreckhise H, Khuu-Duong K *et al.* Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology* 44(4), 896–906 (2006).

16 Myler H, Felx T, Zhu J *et al.* Measuring biotherapeutics with endogenous counterparts and pre-existing antibodies: an interferon case study. *Bioanalysis* 6(8), 1113–1122 (2014).

17 Shankar G, Devanarayan V, Amaravadi L *et al.* Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J. Pharma. Biomed. Anal.* 48 (2008) 1267–1281.

18 Krishna M, Palme H, Duo J *et al.* Development and characterization of antibody reagents to assess anti PEG IgG antibodies in clinical samples. *Bioanalysis* (In Press).