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Anti-PEG Antibodies From mRNA COVID-19 Vaccines Affect In Vitro Measurements of Pegylated Drug Levels

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ABSTRACT

The recent adoption of mRNA-based technology for vaccine development has led to widespread exposure to new vaccine components, such as polyethylene glycol (PEG), against which antibodies may be made. This study assesses the presence of anti-PEG antibodies in human serum following SARS-CoV-2 vaccination, and if these antibodies could interfere with drug level assessment of PEG containing drugs. Elevated anti-PEG antibody titers with prolonged prevalence were detected in individuals who received the mRNA-1273 vaccine as compared to control samples. Anti-PEG antibody levels were approximately 10-fold higher post-vaccination compared to pre-vaccination in approximately 33% of assessed mRNA-1273 vaccine recipients. Elevated anti-PEG antibody levels persisted for over 6 months. Serum from those who received the BNT162b2 or Ad26.COV2.S vaccines had anti-PEG antibody levels similar to control samples. Serum from individuals with elevated anti-PEG antibodies, regardless of study group, typically showed decreased detection of the pegylated drug, pegfilgrastim. This study demonstrates that anti-PEG antibodies have the potential to interfere with bioanalytical assays used for pharmacokinetic drug measurement during the development of pegylated pharmaceutical products, as well as during the establishment of comparability to pegylated reference products during development of biosimilar drug products.

1 | Introduction

Recent advances in molecular biology and biotechnology have introduced innovations in biological and nanoparticle-based therapeutics. Despite their significant therapeutic advantages, such as high target specificity with limited side effects, challenges such as short half-life, rapid degradation and clearance in clinical settings can overshadow these benefits. Pharmaceutical manufacturers conjugate polyethylene glycol (PEG) to drug products to increase their half-life, which has led to successful development and approval of protein-based products [1, 2].

However, attempts by pharmaceutical sponsors to develop biosimilar alternatives to some pegylated reference products have occasionally failed to meet the necessary regulatory requirements, and we tried to understand why these failures occurred. These efforts focused on the analytical tools used to demonstrate biosimilarity of PEGylated products and on the impact of anti-drug antibodies (ADAs) present in human serum on drug

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Study Highlights

What is the current knowledge on the topic?

Recent research has highlighted the emergence of anti-PEG antibodies following SARS-CoV-2 vaccination, prompting an investigation into how these antibodies impact pegylated drugs.

What question did this study address?

This study explores how increased anti-PEG antibodies, potentially elevated by SARS-CoV-2 vaccinations, affect the pharmacokinetic assessment and serum levels of PEGylated drugs and biosimilars.

What does this study add to our knowledge?

It provides new insights on how vaccination-induced anti-PEG antibodies may influence the pharmacokinetics of PEGylated drugs. By analyzing serum samples from different periods and vaccination statuses, it offers data on how these antibodies might distort drug concentration measurements, affecting biosimilar development and approval.

How might this change clinical pharmacology or translational science?

The findings suggest modifying pharmacokinetic evaluations to include vaccine history and antibody testing, potentially refining dosing and safety guidelines. For translational science, this study highlights the necessity for improved analytical methods that accommodate antibody variability, advocating for updated drug development standards.

pharmacokinetics (PK). The presence of pre-existing antibodies to some components of protein therapeutics has long been a concern for drug developers and policymakers [3–5]. Further, the ubiquitous presence of PEG, in foods, cosmetics, and over the counter (OTC) products, has been implicated as a source of exposure that results in the development of anti-PEG antibodies [6, 7].

Standard practice is to use pooled normal human serum as assay matrix for development of in vitro methods used to assess drug concentration and immunogenicity [8]. During attempts to measure the concentrations of a reference pegylated granulocyte colony-stimulating factor (G-CSF) product, pegfilgrastim (Neulasta), and its biosimilars using different in vitro methods, we observed that concentrations measured using pooled human serum post-pandemic were significantly lower than those measured from pre-pandemic serum (Table S1). These findings led to the investigations reported here.

Given the use of human serum during drug development, increases in the occurrence of anti-PEG antibodies in the population, if not accounted for, could potentially impact experimental results during the development of pegylated drugs and biosimilars. With the rise of lipid nanoparticle—mRNA (LNP-mRNA) based technology, such as that used for manufacturing some SARS-CoV-2 vaccines, it was critical to establish whether systemic exposure to PEG in a vaccine could increase the prevalence of anti-PEG antibodies in the population. Recent studies reporting the development of anti-PEG antibodies in association with SARS-CoV-2 vaccination have emerged and called for an examination of the effect of such antibodies on pegylated drugs [9–11].

Based on our preliminary results and literature reports, we hypothesized that SARS-CoV-2 vaccinations containing PEG can result in increased anti-PEG antibodies, interfering with measurement of PEGylated products. To test this hypothesis, we designed a series of experiments using serum from healthy individuals that were collected either pre-pandemic or after 2020 from either self-reported unvaccinated individuals or those who received one of three SARS-CoV-2 vaccines. The results of these experiments and their potential impact on PK measurement as well as serum drug levels are presented.

2 | Materials and Methods

2.1 | Study Design

This study utilized commercially available human serum samples collected either at a single time point or serially over time following SARS-CoV-2 vaccination. Samples were grouped based on vaccination status, including recipients of mRNA-1273, BNT162b2, Ad26.COV2.S, or unvaccinated/pre-pandemic controls. For the single-time-point cohorts, the median intervals between the first vaccine dose and serum collection were 56 days (range: 35–122 days) for BNT162b2 recipients and 54 days (range: 41–126 days) for mRNA-1273 recipients. All human serum samples used in this study were commercially obtained and fully de-identified prior to receipt by investigators. No personally identifiable information was accessible to study investigators. Available donor information included age, sex, ethnicity, collection location, vaccination status, and collection date when provided by the vendor (Tables 1 and S4).

The study consisted of three experimental phases. First, anti-PEG antibody levels were assessed in serum collected at single time points using two commercially available anti-PEG ELISA assays to determine if similar results would be obtained with different assays. Second, serial serum samples collected following vaccination were analyzed to evaluate the magnitude and persistence of anti-PEG antibody responses over time. Third, selected serum samples were used in pegfilgrastim spike-and-recovery experiments to determine whether anti-PEG antibodies interfered with in vitro detection of pegylated drug products using ligand-binding and cell-based bioanalytical assays.

2.2 | Human Serum Samples

Human serum samples were obtained from six commercial sources: Access Biologicals, Vista, CA (Vendor 1), RayBiotech, Peachtree Corners, GA (Vendor 2), Boca Biolistics, Pompano Beach, FL (Vendor 3), BioIVT, Westbury, NY (Vendor 4), Precision for Medicine, Frederick, MD (Vendor 5), and Innovative Research, Novi, MI (Vendor 6). Serum samples were received on dry ice and initially stored at −80°C. Later, samples

TABLE 1 | Demographic profile of individuals whose serum was collected at a single time point.

Characteristics no. (%)	Pre-pandemic or unvaccinated	BNT162b2	mRNA-1273	Ad26.COV2.S
Number	138	54	58	10
Age, median (IQR), year	36 (25.3–53.0)	53 (39.9–71.3)	53 (39.0–67.0)	39 (26.8–46.3)
Sex				
Men	74 (54)	27 (50)	29 (50)	5 (50)
Women	64 (46)	27 (50)	29 (50)	5 (50)
Race				
White	96 (70)	39 (72)	38 (66)	5 (50)
Black or African American	40 (29)	11 (20)	13 (22)	4 (40)
Asian	1 (< 1)	4 (8)	5 (9)	
Other	1 (< 1)		2 (3)	1 (10)
Hispanic or Latino ethnicity	45 (33)	10 (19)	7 (12)	4 (40)
State of collection or area of residence				
Florida	50			
Georgia		11	17	
Louisiana		17	15	
Tennessee		8	4	
Not disclosed	88	18	22	10

were thawed at room temperature and aliquoted into single-use volumes (to avoid repeated freeze–thaw cycles) and stored at −80°C until use in assays. Sample sizes were determined based on the availability of commercially sourced serum samples meeting study inclusion criteria, including vaccine type, collection time points, and longitudinal sample availability. Additional details regarding serum sample categorization and longitudinal collections are provided in the text of Supporting Information as well as summarized in Tables 1 and S4.

2.3 | Anti-PEG Antibody Detection

We used two commercial ELISA assays to detect human IgG anti-PEG antibodies. The anti-poly-PEG assay (Alpha Diagnostic Intl Inc., TX) uses a poly-methoxy PEG-40K-BSA conjugate as the capture antigen. The anti-mono-PEG assay (Life Diagnostic Inc., PA) uses a mono-methoxy PEG-20 K-BSA conjugate [12, 13]. The same chimeric monoclonal antibody was used for both assays' standard curves, with results read using a Spark microplate reader (Tecan, Switzerland). Additional methodological details are provided in Appendix S1.

2.4 | G-CSF Ligand Binding Assay

The G-CSF ligand binding assay utilizes a commercial ELISA kit (R&D Systems, Minneapolis, MN). Pegfilgrastim was diluted in pooled-gender human serum (negative for anti-PEG antibodies), establishing a calibration curve from 200 to 6000 pg/mL. The assay employs anti-G-CSF antibodies for capture and detection. To assess the impact of potential anti-PEG antibodies, individual serum samples were diluted in pooled serum (negative for anti-PEG antibodies), then incubated with pegfilgrastim at 2000 pg/mL for 20 min at room temperature. The assay was completed following incubation. Additional methodological details are provided in Appendix S1.

2.5 | G-CSF-Receptor Cell-Based Assay

Pegfilgrastim was diluted in pooled human serum (negative for anti-PEG-antibodies) to create a calibration curve from 1000 to 60,000 pg/mL. For G-CSF assay performance, serum samples were diluted 1:5 in pooled serum, mixed with pegfilgrastim, and incubated for 30 min at 4°C to bind anti-PEG antibodies. AML-193 cells (CRL-9589, ATCC, Manassas, VA) were washed and seeded at 100,000 cells/well, then incubated with pegfilgrastim in serum. Post-incubation, cells were stained with an anti-PEG monoclonal antibody, then Alexa Fluor 488-conjugated anti-IgG antibodies. After fixation, data were collected via flow cytometry and analyzed [14]. Additional methodological details are provided in Appendix S1.

2.6 | Anti-SARS (CoV-2) Antibody Titers

Concentrations of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Spike (trimer) IgG antibodies were measured in diluted human serum (1:100–1:4000) using Human SARS-CoV-2 Spike (Trimer) IgG ELISA Kit, BMS2325 (Invitrogen, Waltham, MA), according to the manufacturer's

instructions. Each plate included pre-pandemic pooled human serum (diluted 1:100) and a kit-provided positive control. Washing was completed using a BioTek ELx50 plate washer. Data were collected using a Spark microplate reader (Tecan, San Männedorf, Switzerland).

2.7 | Depletion of Anti-PEG IgG

Polystyrene beads linked with methoxy-terminal PEG 2000 (PS50-PG-1; Nanocs Inc., Boston, MA) were used to deplete anti-PEG IgG from serum samples. Depleted serum was then tested with G-CSF ELISA and cell-based assays. Additional methodological details are provided in Appendix S1.

2.8 | Competitive Assay to Determine Anti-PEG Antibody Saturation Limit

Three individual sera were used to evaluate the blocking potential of anti-PEG antibodies present. Spike amounts were based on average maximum concentration (C_{max}) of subcutaneously administered 6 mg pegfilgrastim (160 ng/mL) and 2 mg pegfilgrastim (36 ng/mL). Pooled human serum negative for anti-PEG antibodies was used as the matrix control for calibration standards and quality control samples. Unspiked anti-PEG ADA negative donor serum served as a baseline negative control. Additional methodological details are provided in Appendix S1.

2.9 | Statistical Analysis

All in vitro assays assessed using the Spark microplate reader had accepted analytical runs initially analyzed in Excel. Data were exported to GraphPad Prism and standard curve data were fitted to a four-parameter logistic curve. For acceptance, standards were within ±20% of the expected value, and the standard curve R^2 must be ≥ 0.990. Statistical differences between groups were determined using one-way ANOVA with Tukey's multiple comparisons test. For correlation studies, simple linear regression analysis with best-fit curve was used. All statistical analyses were performed using GraphPad Prism 9.1.2.

3 | Results

3.1 | SARS-CoV-2 Vaccination and Anti-PEG Antibody Development

Initially, we determined the prevalence of anti-PEG antibodies in individuals vaccinated against SARS-CoV-2, as well as in unvaccinated individuals, using serum samples obtained from six different commercial vendors (Table S1). We used two anti-PEG antibody assays to assess these values, one detecting poly-methoxy-PEG (anti-poly-PEG), Figure 1A, other detecting mono-methoxy-PEG (anti-mono-PEG), Figure 1B, specific antibodies. All four groups included individuals with high levels of anti-PEG antibodies, with the median anti-poly-PEG titer of 5911 ng/mL (control); 12,373 ng/mL (mRNA-1273); 6158 ng/mL (BNT162b2); and 6473 ng/mL (Ad26.COV2.S), Figure 1A, Table S2. Those receiving the mRNA-1273 vaccine had significantly higher ($p < 0.01$) anti-PEG antibodies compared to all other groups for anti-poly-PEG and anti-mono-PEG antibodies (Figure 1A,B). No correlation was found between development of anti-SARS-CoV-2 antibodies and anti-PEG antibodies (Figure S1).

3.2 | Correlation Between Anti-PEG Antibodies and Detection of Pegfilgrastim in Serum Samples

To understand if anti-PEG antibodies impact the measured concentration of pegylated drugs, we spiked serum samples with known concentrations of pegfilgrastim and measured pegfilgrastim concentrations using two assays. One assay, developed in our laboratory, used cells expressing the G-CSF receptor to assess drug detection (cell-based assay, Figure 1C) with a spike concentration of 7000 pg/mL. The other assay, commonly used for pegfilgrastim biosimilar submissions, used a spike concentration of 2000 pg/mL (G-CSF ELISA, Figure 1D). The detection of spiked pegfilgrastim concentrations varied for all four groups. In the cell-based assay, the median detected was 7696 pg/mL (control); 6246 pg/mL (mRNA-1273); 7566 pg/mL (BNT162b2) and 7377 pg/mL (Ad26.COV2.S.), with the mRNA-1273 group being significantly lower than all other groups (Figure 1C, Table S3). In the G-CSF ELISA, the median detected was 2078 pg/mL (control); 1952 pg/mL (mRNA-1273); 2157 pg/mL (BNT162b2) and 2264 pg/mL (Ad26.COV2.S.), with the mRNA-1273 group being significantly different from all other groups (Figure 1D, Table S2). Furthermore, when pegfilgrastim concentrations were measured using the cell-based assay, approximately 15% of individuals vaccinated with mRNA-1273 exhibited greater than 50% loss of drug detection versus spiked concentration (Figure 1C). All other groups had no more than 2% of individuals with loss of drug detection falling below 50%. The correlation between anti-poly-PEG antibodies and drug detection for individual samples is shown by group (Figure 2, Table S3). A trend of increasing anti-poly-PEG antibody titer in individual serum was significantly associated with decreased drug detection. However, some individuals with low or moderate levels of anti-poly-PEG antibodies still demonstrated decreased concentrations as measured by the cell-based assay.

3.3 | Serial Serum Samples From mRNA-1273 Vaccination Are Associated With Prolonged Anti-PEG Antibody Response and Interference With Drug Detection

Anti-poly-PEG antibodies were measured in serial serum sets collected following vaccination obtained from three different vendors (Table S4). The first set consisted of serum samples from 15 individuals vaccinated with mRNA-1273 and collected at six different time points (Figure 3A; Table S5). Although the anti-poly-PEG antibody response varied among individuals, 8/15 of mRNA-1273 recipients showed a greater than 100% increase in anti-poly-PEG antibody response 2 weeks after the second dose of mRNA-1273, and 7/15 showed a greater than 600% increase at 6 weeks following the second vaccination (Figure 3A). Approximately one-third of assessed

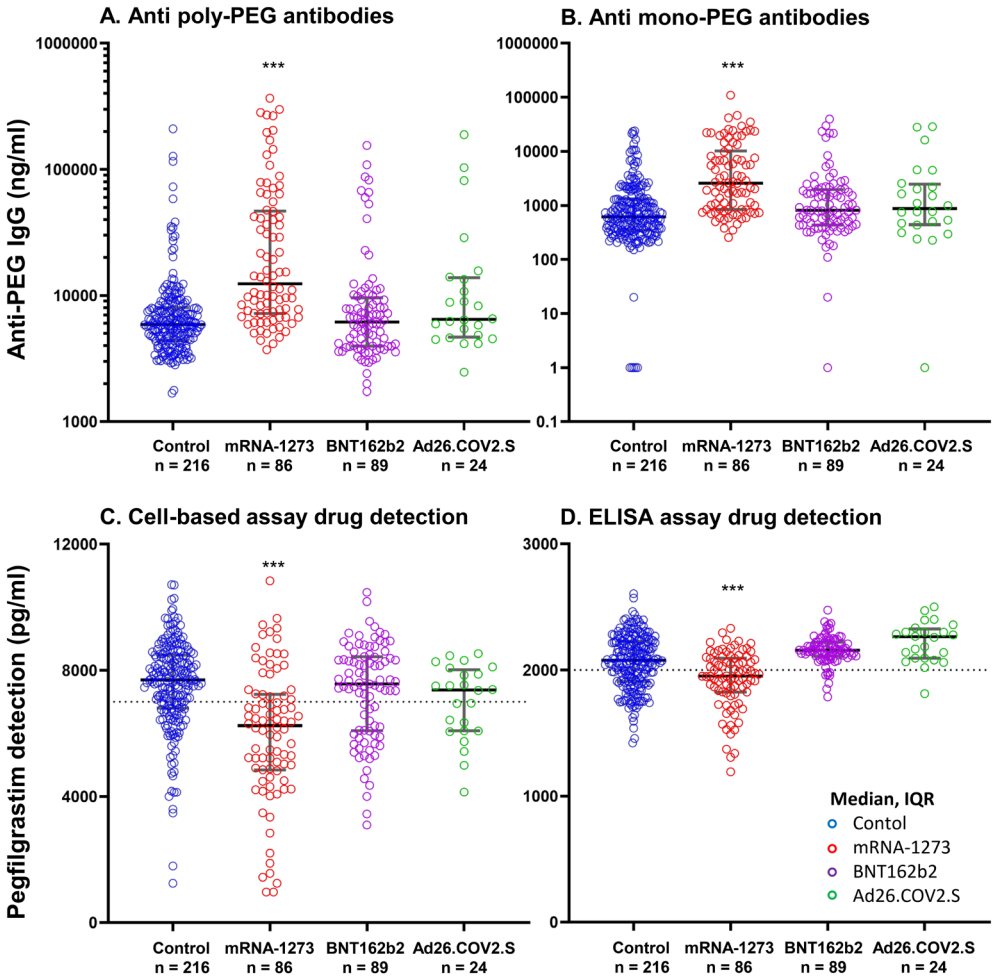

FIGURE 1 | Anti-PEG IgG and pegfilgrastim concentrations following SARS-CoV-2 vaccination in samples collected at a single time point Anti-poly-PEG antibody (A) and anti-mono-PEG antibody (B) titers were measured in serum from unvaccinated/pre-pandemic controls (blue, $n = 216$), mRNA-1273 recipients (red, $n = 86$), BNT162b2 recipients (purple, $n = 89$), and Ad26.COV2.S recipients (green, $n = 24$). G-CSF-Receptor Cell-Based Assay (C) and G-CSF Ligand Binding Assay (D) evaluated pegfilgrastim concentration after spiking at 7000 pg/mL and 2000 pg/mL, respectively (dotted line), utilizing serum samples from the same groups. Each circle represents an individual. The average time between dose #1 and the time of serum collection was 63 days for mRNA-1273, 58 days for BNT162b2, and 78 days for Ad26.COV2.S. Statistical differences between groups were determined by one-way ANOVA with Tukey's multiple comparisons. The bar represents the median with interquartile range (IQR). Significance levels are denoted as follows: ***$p < 0.0001$ versus all other groups. See (Table S2) for statistics. The anti-PEG antibody values shown ng/mL are solely for the purpose of comparing data between the two assay specificities and should not be used to make clinical inferences or conclusion.

mRNA-1273 vaccine recipients demonstrated at least a 10-fold increase in anti-PEG antibody levels relative to baseline following vaccination. The antibodies persisted in 3/15 individuals at >1500% increase, and at >100% in 3/15 individuals at 32 weeks after initial vaccination. Some (5/15) mRNA-1273 vaccinated individuals did not have increased anti-poly PEG antibodies versus baseline through the 32 weeks. Sera obtained from other vendors also showed increasing anti-poly PEG antibody levels following vaccination with mRNA-1273, although the final serum collection point for these individuals occurred 6–12 weeks post-vaccination (Figure 3B) so longer-term persistence could not be assessed.

The group receiving BNT162b2 vaccinations did not have increased titers over time; although three individuals had pre-existing anti-poly-PEG antibodies prior to initial vaccination, and their antibody levels increased after each dose (Figure 3C,D; Table S5).

Other than those with pre-existing anti-poly-PEG antibodies, all others receiving BNT162b2 vaccinations did not mount a measurable anti-poly-PEG antibody response. Unlike mRNA-1273 and BNT162b2, the Ad26.COV2.S serum set consisted of only one vaccine dose; however, this vaccine does not contain any PEG. As evident from Figure 3E, two individuals receiving Ad26.COV2.S had high pre-existing anti-poly-PEG antibody levels. One of 14 Ad26.COV2.S vaccinated individuals developed a significant anti-PEG antibody response measurable 2 weeks after vaccine administration. The effect of anti-poly-PEG antibodies on in vitro detection of pegfilgrastim for the longitudinal samples is shown in Figure 4. As measured by cell-based assay (Figure 4A,C,E) and G-CSF ELISA (Figure 4B,D,F), the presence of anti-poly-PEG antibodies corresponded to decreases in drug detection. For mRNA-1273 vaccinated individuals, the effect of anti-poly-PEG antibodies on drug detection persisted through 32 weeks. Those receiving either BNT162b2 or Ad26.COV2.S did not show substantial loss of

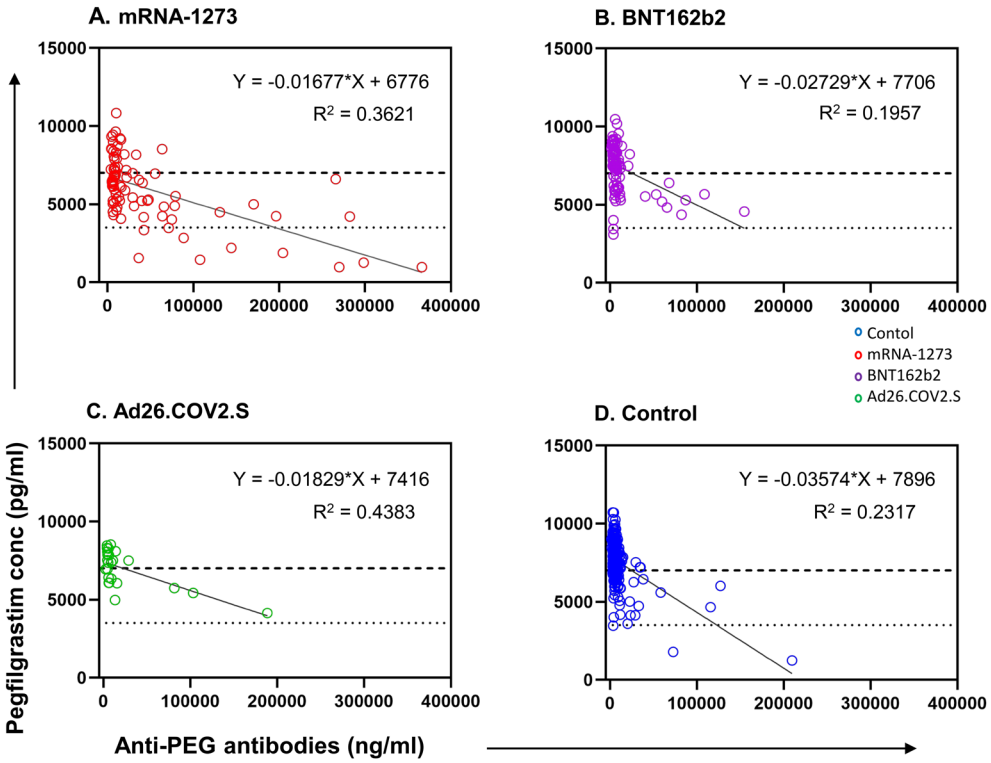

FIGURE 2 | Correlation between anti-PEG antibodies and drug detection in a serum obtained at a single time point. Simple linear regression analysis with best-fit curve was used to examine the relationship between anti-poly-PEG IgG titer and pegfilgrastim detection in spiked human serum. Drug spike and detection measured by G-CSF-receptor cell-based assay. Dotted line at 3500 pg/mL represents 50% loss of drug detection; dashed line at 7000 pg/mL represents the spiked drug concentration. Each circle represents a serum from an individual. Corresponding statistical analysis in supplemental (Table S4) mRNA-1273, $n = 86$; (B) BNT162b2, $n = 89$; (C) Ad26.COV2.S, $n = 24$; (D) pre-pandemic or unvaccinated control, $n = 218$. The average time between dose #1 and time of serum collection: MRNA-1273 63 days, BNT162b2 58 days, Ad26.COV2.S 78 days.

drug detection, with the exception of one individual that developed anti-poly-PEG antibodies in the Ad26.COV2.S cohort.

Serial serum collection for BNT162b2 was only measured through 6 or 12 weeks, based on vendor sample availability. Therefore, we obtained and assessed additional serum from BNT162b2 vaccinated individuals that extended to greater than 30 weeks post-vaccination and included later booster vaccination (Figure S2). No significant change in the anti-poly-PEG antibody response was noted.

3.4 | Anti-PEG Antibodies Are the Cause of Decreased Detection of Pegfilgrastim

To determine if anti-PEG antibodies were responsible for loss of detection, we selected individuals from each treatment group, used beads to deplete any anti-PEG antibodies, then spiked samples with drug to assess drug detection. We show that when anti-PEG antibodies were removed from samples with low drug detection, drug detection was restored (Figure 5A,B). Further, we show in Figure 5C,D that if serum with no anti-PEG antibodies is treated with depletion beads, no change in detection occurs. If serum with anti-PEG antibodies or serum spiked with a monoclonal anti-PEG antibody is tested, both demonstrate anti-PEG antibody depletion restoring drug detection. This shows that blocked drug detection by anti-PEG antibodies can be overcome in cell culture.

To determine if anti-PEG antibodies could impact measurement of drug product at clinically relevant concentrations, we conducted a competitive assay in which increasing amounts of pegfilgrastim were added to serum samples and then measured in drug detection assays. Amount of spiked drug used in this assay correlated to the C_{max} of two doses used in clinical trials for biosimilar pegfilgrastim products, 2 and 6 mg, with mean corresponding C_{max} of ~40 and ~160 ng/mL, respectively [15, 16]. Three individuals were selected based on the presence of high, intermediate, or low anti-poly-PEG antibody levels, with all three having less than 50% detection of drug in the cell-based assay (Figure 6A).

Results for the cell-based assay (Figure 6B) for individual #1 show near 100% drug detection occurred when serum was spiked with 160 ng/mL of pegfilgrastim; however, when 40 ng/mL was added, pegfilgrastim detected was under 50%. Individual #2 achieved only 70% pegfilgrastim detection at 160 ng/mL and < 25% drug detection at 40 ng/mL spike. Donor #3 achieved full detection of pegfilgrastim at 80 ng/mL. When using G-CSF ELISA (Figure 6C), we found all individuals' detection returned to 80%–120% detection when spike concentration was at least 8 ng/mL.

4 | Discussion

This study evaluated the presence of anti-PEG antibodies in individuals vaccinated with a SARS-CoV-2 vaccine or unvaccinated, and determined if anti-PEG antibodies could

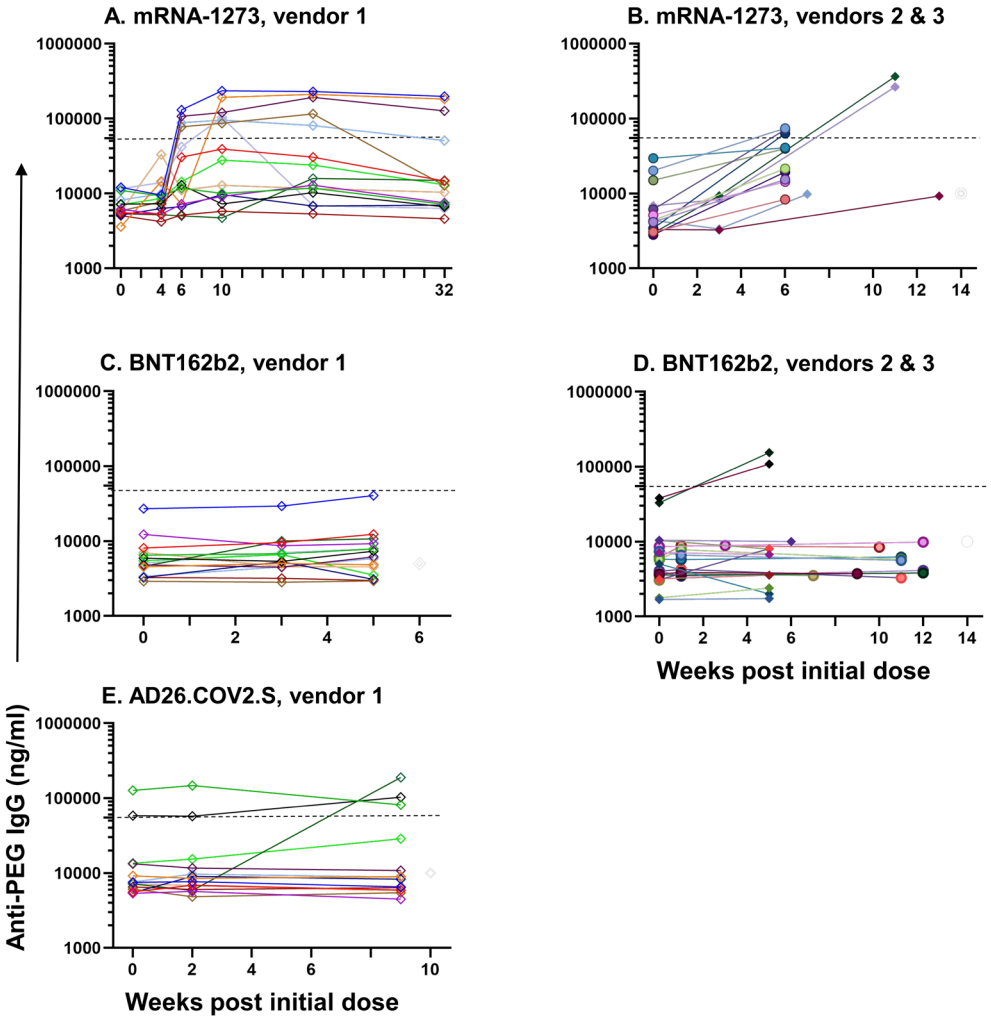

FIGURE 3 | Detection of anti-PEG antibodies in serial samples. Anti-poly-PEG antibody titers (ng/ml) were measured for several vaccine cohorts. (A) mRNA-1273, $n = 15$, vendor #1. Serum was collected at Week 0 (pre-first dose), Week 4 (pre-second dose), and 6, 10, 19, and 32 weeks post-first vaccination. (B) mRNA-1273, $n = 14$, vendors #2 and 3. Serum collected at Week 0 (pre-first dose) and varied post-vaccination intervals. (C) BNT162b2, $n = 15$, vendor #1. Serum collected at Week 0 (pre-first dose), three, and 5 weeks post-first vaccination. (D) BNT162b2, $n = 20$, vendors #2 and 3 collected at Week 0 and various post-vaccination time points. (E) AD26.COV2.S, $n = 14$, vendor #1. Serum collected at Week 0 (pre-vaccination), Weeks 2, and 9 post-vaccination. The line color in each panel represents a unique individual. The dashed line represents the titer correlated to a 50% reduction in drug detection. See (Table S5) for statistics.

impact detection of pegfilgrastim in spiked serum samples. Subsequently, serial serum samples were evaluated to investigate the magnitude and persistence of anti-PEG antibodies over time.

Two of the tested vaccines (mRNA-1273 and BNT162b2) contained a PEG component, whereas Ad26.COV2.S did not. Consistent with previous reports, some of the individuals vaccinated with mRNA-1273 had anti-PEG antibody responses [10, 17], statistically greater than found in control serum. BNT162b2 vaccinated individuals did not show increased anti-PEG antibodies versus control serum, also consistent with prior studies.

The cause for differences in anti-PEG antibody response is unclear; however, at least two factors could play a role. First, the originally approved mRNA-1273 vaccine was administered with 100 μg of mRNA per dose, with the amount of PEG present unspecified [18]. The original BNT162b2 had 30 μg of mRNA, containing 0.05 mg of PEG per dose [18]. However, the currently approved formulation of mRNA-1273 now contains 25 μg of mRNA with 0.5 mg of PEG [19] whereas BNT162b2 contains 3 μg of mRNA with 0.005 mg of PEG [20]. If the BNT162b2 vaccine has the same proportion of PEG present in their formulations, the original mRNA-1273 vaccine would have 2 mg of PEG, representing 40 times more PEG than BNT162b2 contained, and more potential antigenic material promoting development of anti-PEG antibodies.

Second, minor differences in PEG structure are associated with each of these vaccines. Additional investigation is needed to determine if these, or other factors, play a role in the induction of anti-PEG antibodies.

Interestingly, two individuals receiving the Ad26.COV2.S vaccine displayed high levels of pre-existing anti-PEG antibodies, while one participant developed them during sampling. This suggests non-vaccine-related anti-PEG antibodies, possibly

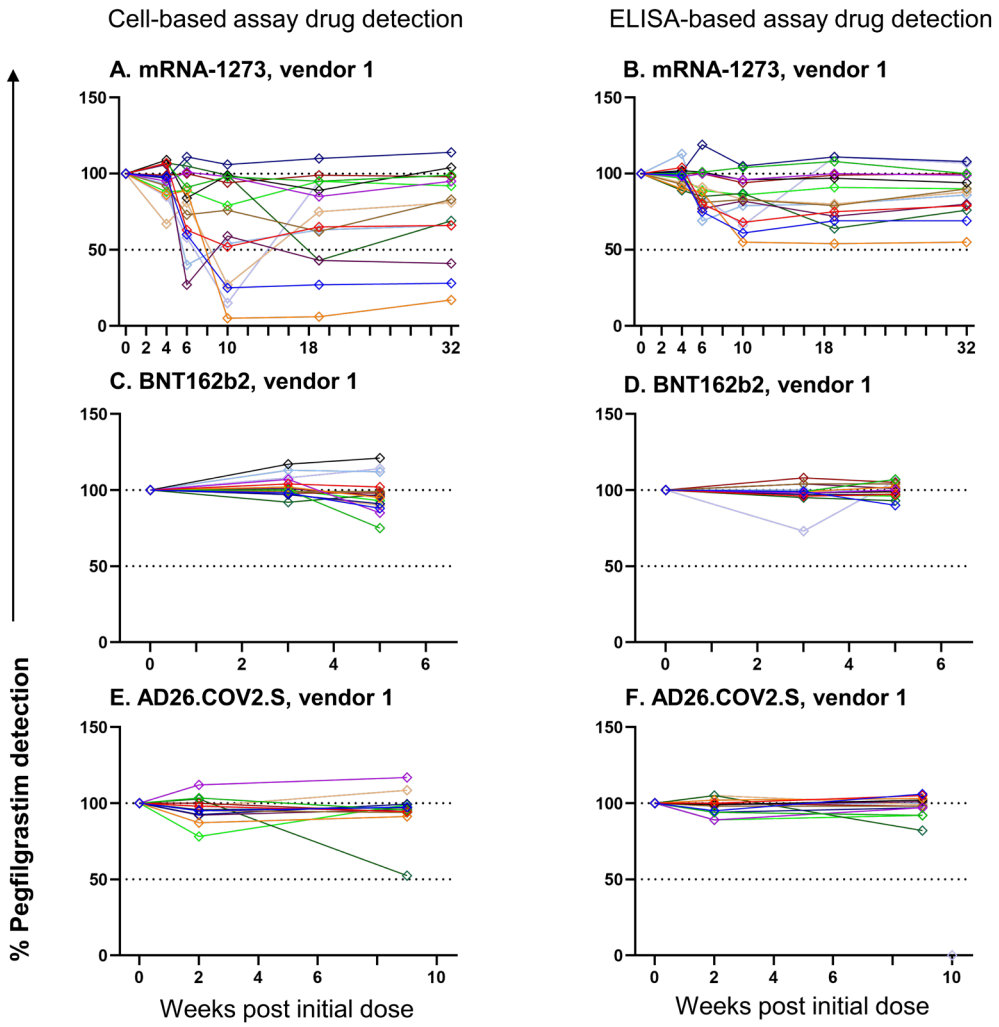

FIGURE 4 | Pegfilgrastim detection in spiked serum from serial samples. G-CSF-receptor cell-based assay measured pegfilgrastim in serum spiked at 7000 pg/mL (A, C, E). G-CSF ligand binding assay measured pegfilgrastim in serum spiked at 2000 pg/mL (B, D, F). A baseline of 100% is established for each individual, representing the detection of spiked pegfilgrastim in serum obtained prior to first vaccination. All subsequent data points are expressed as a percentage of pegfilgrastim detected in relation to the baseline for each individual. All individuals are color-coded consistent with anti-poly-PEG antibody titers in Figure 3A,C,E. Serum from all individuals was collected by vendor #1. For mRNA-1273 ($n = 15$) doses #1 and #2 administered at Week 0 and Week 4, with serum collected at Week 0 (pre-first dose), Week 4 (pre-second dose), and at 6, 10, 19, and 32 weeks post the first vaccination for all individuals (A, B). For BNT162b2 ($n = 15$), dose #1 and #2 were administered at Week 0 and 3. Serum collected at Week 0 (pre-dose), 3, and 5 weeks post the first dose (C, D). For Ad26.COV2.S ($n = 14$) one dose was administered at Week 0. Serum collected at Week 0, prior to dose #1 and Week 2 and 9 prior to dose #2 (E, F).

from foods, cosmetics, or over-the-counter products may be the cause. Alternatively, reports indicate potential cross-reactivity to polysorbate and PEG due to molecular similarity [21], suggesting an immune response to polysorbate-80 in recipients of the Ad26.COV2.S vaccine (for which polysorbate-80 is an excipient).

PEG is not a single unique molecular weight or form; it can have branched or linear forms [22]. Differences in PEG occur during manufacturing, with PEG of the same molecular weight from different sources exhibiting differences in structure and function [23]. To identify as many different anti-PEG antibodies as possible, we used two different commercially available assays for detection: identifying either anti-poly-PEG antibodies or anti-mono-PEG antibodies.

Although the mono-PEG and poly-PEG assays produced different absolute anti-PEG antibody titer ranges, they generally identified similar relative response patterns across individuals. There were also individuals with no anti-PEG antibodies, but with reduced drug detection when pegfilgrastim was spiked into their serum. This suggests that existing anti-PEG antibody assays may not be able to identify all the anti-PEG antibodies that could be present.

Pegfilgrastim was used to assess anti-PEG antibodies' effect on the detection of pegylated drugs in vitro. Pegfilgrastim was selected as our ongoing studies already employed bioanalytical assays to assess drug levels in vitro. We found a correlation between the presence of high anti-PEG antibody titers and decreased in vitro drug detection when a known quantity of drug was spiked into serum samples. Individuals from all four groups had serum positive for anti-PEG antibodies; however, the drug detected was significantly lower for mRNA-1273 as compared to other groups.

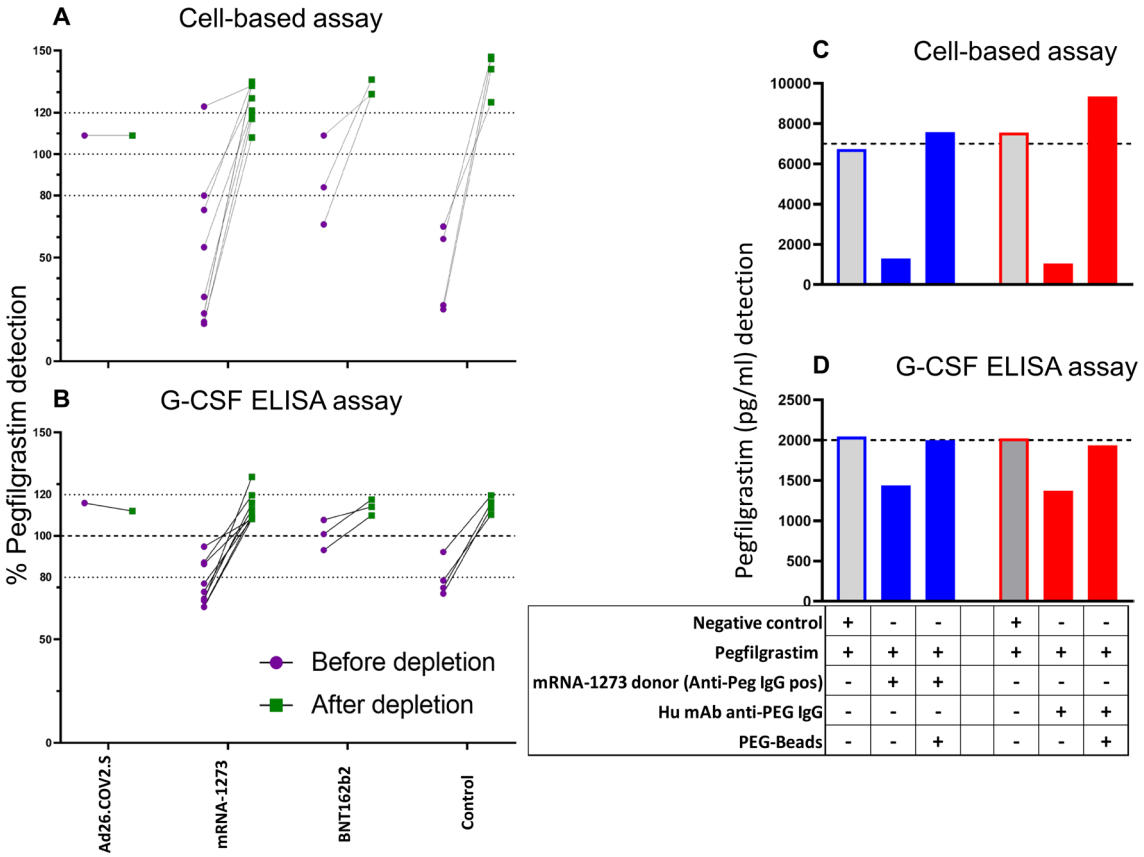

FIGURE 5 | Depletion of anti-PEG antibodies in serum from individuals. Pegylated plastic beads (PEG-2000) were used to deplete anti-PEG antibodies in either vaccinated or control human serum. Each circle connected to a box with a line represents an individual before and after anti-PEG antibody depletion. The G-CSF-receptor cell-based assay measures pegfilgrastim spiked at 7000 pg/mL (A, C) and the G-CSF ligand binding assay measures pegfilgrastim spiked at 2000 pg/mL (B, D). Panels (C, D) show a graphical representation of each depletion condition or treatment when evaluating samples using the specified assay method.

The degree of decrease in drug detection varied between the assays, but the consistency of reduction indicates that drug detection can be impacted. Our use of two different assay methods to detect pegfilgrastim spiked into serum allowed us to demonstrate these differences were not an artifact of a single assay.

Differences in the sensitivity of drug detection between the two assays were observed. Generally, there was less loss of detection in the ELISA-based versus cell-based assay. This is potentially due to the ELISA detecting filgrastim, the G-CSF protein only. No PEG reagents were present in ELISA; therefore, steric hindrance is the implied mechanism of reduced detection. In contrast, the cell-based assay has the cellular receptor present and uses an anti-PEG backbone antibody for detection. It is possible that anti-PEG antibodies blocked the detection antibody, leading to lower overall detection compared to the ELISA. In both cases, detection could be reduced due to steric hindrance from PEG as compared to the small G-CSF protein. Ideally, drug detection would be best measured with a cell-based assay, without PEG reagents, to model anti-PEG antibody effects in vivo.

To understand the persistence of anti-PEG antibodies over time we tested samples collected from the same individuals over time following initial and/or booster vaccination. Anti-PEG antibodies detected in response to two doses of mRNA-1273 persisted in approximately one-third of samples, up to 32 weeks. Individuals receiving the BNT162b2 vaccination series showed no significant changes in anti-PEG antibodies up to 36 weeks after initial administration, or following a booster dose (Figure S2). Interestingly, all groups included individuals with high anti-PEG antibodies that showed minimal effect on pegylated drug detection, implying high titers do not equal drug detection interference. As some antibodies did not impede pegfilgrastim detection, and some pre-existing antibodies did, it is clear that mRNA vaccines are not solely responsible for lost drug detection.

Given the variability in the presence of anti-PEG antibodies versus loss of drug detection, we designed a depletion assay to determine if antibodies were the cause of lost drug detection. Results showed that if drug detection was reduced, it was restored when the serum was pre-incubated with anti-PEG beads, regardless of titer. In contrast, an individual with high anti-PEG antibodies, and no detection loss, was unchanged following depletion. This suggests that serum used for bioanalytical assays of pegylated drugs should be pre-screened for the presence of anti-PEG antibodies.

To address the likelihood that interference from pre-existing anti-PEG antibodies could be overcome through administration

A

Individual	Vaccination Status	Anti-poly-PEG antibody titer (ng/ml)	Anti mono-PEG antibody titer (ng/ml)
1	mRNA-1273	196287	19633
2	Verbally not vaccinated (2022)	72764	5890
3	mRNA-1273	20247	5405

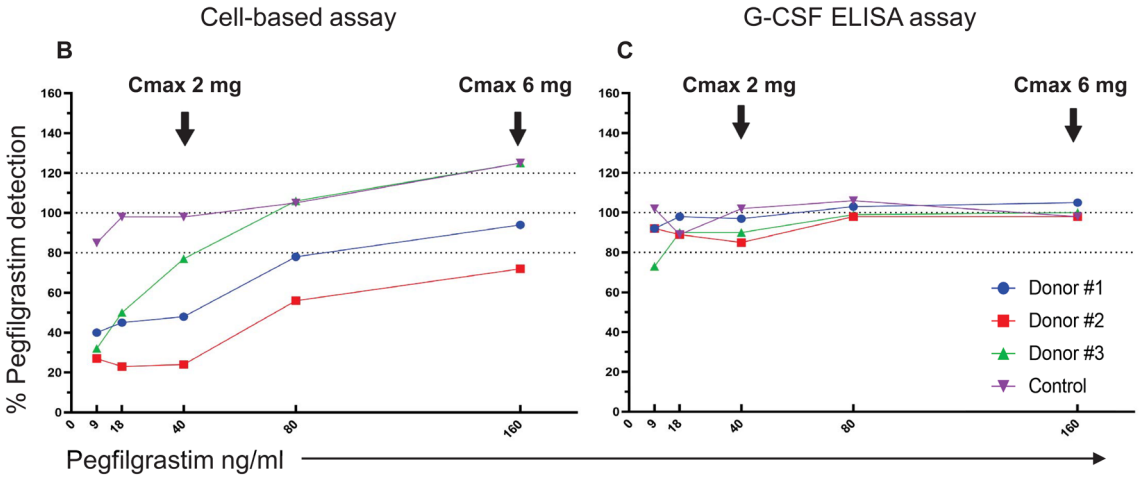

FIGURE 6 | Anti-PEG antibody saturation with pegfilgrastim drug product. Vaccination status and anti-PEG antibody titers measured for poly- and mono-PEG antibodies are shown for the serum from each individual (A) used in plots (B, C). To determine if the anti-PEG antibodies could be overcome through drug saturation, we used the G-CSF-receptor cell-based assay (B) and G-CSF ligand binding assay (C) to measure pegfilgrastim concentrations in serum spiked with increasing concentrations of pegfilgrastim. Data are presented as the proportion of drug detected relative to the amount of the spike. Dashed lines are shown for the concentration range of 100% ±20% to show when the amount detected achieved the range typically used in PK assays with the average concentration for drug product testing doses (2 and 6 mg) shown in each figure. Pegfilgrastim spiked into serum from each individual at 9, 18, 40, 80, 160, 320 ng/mL. The average C_{max} of subcutaneously administered 2 and 6 mg pegfilgrastim are 40 and 160 ng/mL, respectively.

of additional drug product, we conducted a competitive assay with serum from three individuals. We showed all individuals had restored detection when doses of 8 ng/mL or greater were added to cell culture with the ELISA assay. For the cell-based assay, two of the individuals had detection within 80%–120% when 80 ng/mL was added, but one individual did not reach 80% even when 160 ng/mL was added to their serum. These results suggest that anti-PEG antibodies may be overcome similar to insulin dosing to overcome anti-insulin antibodies [24]. However, during biosimilar development, clinical trials are sometimes conducted with subclinical doses. For example, MYL-1401H, a pegfilgrastim biosimilar, only 2 mg was used instead of 6 mg with C_{max} of products ranging from 34.2 to 36.7 ng/mL [16]. This is significantly lower than standard clinical dose (mean C_{max} of 160 ng/mL) [15]. The lower dose may be more sensitive to effects of anti-PEG antibodies induced by vaccination and could increase variability jeopardizing PK similarity. Increased variability is observed at lower concentrations compared to when higher concentrations of drug are present, even when not considering anti-PEG antibodies. Also, our experiments were conducted with a set volume of serum; if individuals are producing anti-PEG antibodies, they could produce more antibody when dosed with a pegylated drug, resulting in altered PK assessment.

With the global adoption of the mRNA-based vaccine technology, the prevalence of the anti-PEG antibodies is expected to increase. This suggests that pre-screening of clinical study volunteers for anti-PEG antibodies may be needed when testing pegylated drugs. The inability of existing screening assays to detect a broad repertoire of anti-PEG antibodies was demonstrated in our study. This highlights the need for multiple assay approaches to fully assess the presence and effect of anti-PEG antibodies.

Anti-PEG antibodies are likely diverse, potentially having varied in vitro and in vivo effects on pegylated drugs; potentially impacting immunogenicity assessment and depending on their affinity for PEG, altering PK and/or clearance. The impact on drug clearance is critical as anti-PEG antibodies have been implicated in increased clearance of pegylated therapeutics through the accelerated blood clearance phenomenon [25–27]. Therefore, it is important to not only detect the presence but also their impact for each pegylated drug product.

Prior reports have demonstrated pre-existing anti-PEG antibodies, although their clinical and bioanalytical relevance remains context-dependent and may vary by therapeutic product, antibody characteristics, and assay system [28–30]. Here, we had

two individuals vaccinated with Ad26.COV2.S, and at least 10 unvaccinated individuals with high pre-existing antibodies. In some cases, the pre-existing antibodies impacted our ability to detect spiked pegfilgrastim. Although few people have documented cases of anaphylaxis to PEG [31, 32], monitoring for anti-PEG antibodies is important for pegylated drug development programs.

This study only evaluated pegfilgrastim for the effect of anti-PEG antibodies on drug detection. Other pegylated drug products will vary in PEG structure and size, and in immunogenicity observed for the product. These variations could impact anti-PEG antibody effects on drug detection and clearance. Further, we did not address the potential impact of anti-PEG antibodies on the clearance or clinical drug efficacy. However, if physicians noted an unexplained loss of efficacy for a PEGylated drug in a patient, the presence of anti-PEG antibodies could be considered as a rule out.

In summary, this study showed that anti-PEG antibodies could interfere with in vitro measurement of pegylated drug concentrations, thereby potentially impacting PK assessment and immunogenicity testing in drug development. Based on the results of this study, the presence of anti-PEG antibodies in serum samples should be considered during preclinical and clinical studies for the development of pegylated drugs. In addition, new assays and approaches may be necessary to accurately detect and interpret the effects of anti-PEG antibodies on the assessment of pegylated drugs.

Author Contributions

E.A.S., K.E.H., J.L.W., J.A.F., and R.R. wrote the manuscript. K.E.H., E.A.S., and J.L.W. designed the research. E.A.S., Z.L., R.E.B., K.E.H., A.P.F., and J.M.S. performed the research. E.A.S., Z.L., R.E.B., K.E.H., J.M.S., and J.A.F. analyzed the data.

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Disclosure

Disclaimer: The findings and conclusions in this article have not been formally disseminated by the U.S. Food and Drug Administration (FDA) and should not be construed to represent any Agency determination or policy. The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the US Department of Health and Human Services.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** Supplementary Methods. **Figure S1:** Titer correlation: anti-poly-PEG IgG versus anti-SARS CoV IgG. **Figure S2:** Serial analysis of anti-poly-pPEG antibodies in serum from BNT162b2-vaccinated individuals. **Table S1:** Quantitation of biosimilars using serum from 2019 versus 2021. **Table S2:** Statistics for experimental data of sera collected at single time points. **Table S3:** Statistics for correlation between anti-PEG antibody titer and pegfilgrastim detection. **Table S4:** Demographic profile of individuals whose serum collected at serial time points. **Table S5:** IQR of anti-PEG antibodies collected at serial time points (Vendor #1).