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Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the General Population

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Abstract

Circulating antibodies (Ab) that specifically bind polyethylene glycol (PEG), a biocompatible polymer routinely used in protein and nanoparticle therapeutics, have been associated with reduced efficacy of and/or adverse reactions to therapeutics modified with or containing PEG. Unlike most antidrug antibodies that are induced following initial drug dosing, anti-PEG Ab can be found in treatmentnaïve individuals (i.e., individuals who have never undergone treatment with PEGylated drugs but most likely have been exposed to PEG through other means). Unfortunately, the true prevalence, quantitative levels, and Ab isotype of pre-existing anti-PEG Ab remain poorly understood. Here, using rigorously validated competitive ELISAs with engineered chimeric anti-PEG monoclonal Ab standards, we quantified the levels of anti-PEG IgM and different subclasses of anti-PEG IgG (IgG1−4) in both contemporary and historical human samples. We unexpectedly found, with 90% confidence, detectable levels of anti-PEG Ab in ~72% of the contemporary specimens (18% IgG, 25% IgM, 30% both IgG and IgM). The vast majority of these samples contained low levels of anti-PEG Ab, with only ~7% and ~1% of all specimens possessing anti-PEG IgG and IgM in excess of 500 ng/mL, respectively. IgG2 was the predominant anti-PEG IgG subclass. Anti-PEG Ab's were also observed in ~56% of serum samples collected during 1970−1999 (20% IgG, 19% IgM, and 16% both IgG and IgM), suggesting that the presence of PEG-specific antibodies may be a longstanding phenomenon. Anti-PEG IgG levels demonstrated

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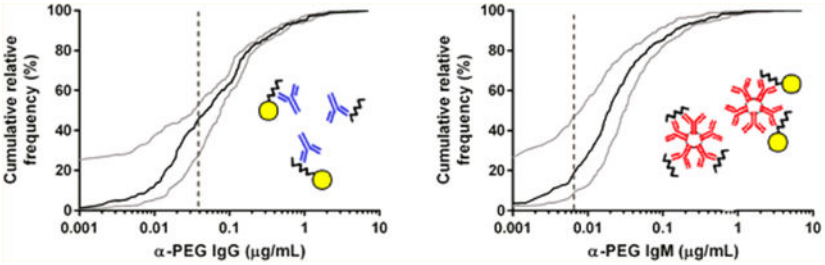
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03437. Anti-PEG Ab ELISA competition and parallelism, linear regression analysis of anti-PEG Ab levels with age, frequencies and cumulative frequencies of anti-PEG IgG1−4 for historical serum samples, frequencies of total IgG and IgM for contemporary plasma and historical serum samples, binding kinetics for chimeric anti-PEG IgG and IgM, anti-PEG Ab ELISA assay details (PDF)

The authors declare no competing financial interest.

correlation with patient age, but not with gender or race. The widespread prevalence of pre-existing anti- PEG Ab, coupled with high Ab levels in a subset of the population, underscores the potential importance of screening patients for anti-PEG Ab levels prior to administration of therapeutics containing PEG.

Graphical Abstract



Immunogenicity encompasses the entirety of innate, humoral, and cellular immune responses against therapeutic molecules and is frequently associated with the induction of antibodies that directly bind to therapeutic molecules (i.e., antidrug antibodies) after the initial or repeated administration of the drug. Both innate and adaptive immune responses can result in decreased efficacy or treatment failure due to either direct neutralization of the therapeutic molecules[1] or inadequate drug dosing at target cells/tissues because of altered pharmacokinetics and biodistribution.[2] Worse, hypersensitivity reactions may lead to adverse or even fatal reactions to a therapy.[3,4] While major strides have been made to reduce immunogenicity, such as development of humanized or fully human monoclonal antibodies, immunogenicity continues to be a major concern for safety and efficacy of many novel drug products.[5]

An emerging class of antidrug antibodies are those that specifically recognize and bind poly(ethylene glycol) (PEG), a synthetic polymer routinely used both as an excipient in pharmaceutical formulations and also as a polymer conjugate to improve the stability and circulation kinetics of protein drugs and nanocarriers.[6,7] PEG is a hydrophilic and highly flexible polymer comprised of repeating subunits of ethylene glycol ($[-O-CH_2-CH_2-]_n$). Because densely PEG-grafted surfaces are exceptionally resistant to protein adsorption,[7–9] PEG has long been assumed to possess little to no immunogenicity, and PEGylation has even been used to mitigate the immunogenicity of therapeutic proteins.[10] Although the potential immunogenicity of PEG was underappreciated at the time, Richter and Akerblom in 1983 reported the possibility that PEGylated proteins, unlike free PEG that generated minimal responses, can actually induce PEG-specific antibodies.[11] Later on, various research groups observed that repeat doses of otherwise long-circulating nanocarriers modified with PEG or PEG-containing molecules were rapidly cleared by mononuclear phagocyte system (MPS) cells in rodent and other animal models.[12,13] These early *in vivo* observations were eventually categorized into a phenomenon termed the accelerated blood clearance (ABC) effect, whereby the first dose of a PEG-containing agent induces anti-PEG antibodies (anti-PEG Ab) that then opsonize and facilitate rapid elimination of subsequent

doses of PEGylated therapeutics.[14] In nearly all animal studies, anti- PEG Ab responses were largely mediated by IgM class antibodies and were transient in nature.[15,16]

Growing evidence suggests that human patients can also generate immune responses to PEG-modified therapeutics, with significant effects on clinical outcomes. The presence of anti- PEG Ab has been associated with rapid clearance of various PEGylated proteins in clinical trials,[17,18] as well as anaphylactic or hypersensitivity reactions after the administration of PEG- containing formulations.[19,20] In contrast to most antidrug antibodies, an important feature of human anti-PEG Ab responses is that these PEG-binding Ab's can be found even in "treatment-naïve" individuals (i.e., individuals who have never undergone treatment with PEGylated drugs), presumably due to prior exposure to PEG. This phenomenon is commonly referred to as "pre-existing" anti-PEG Ab.[16] Indeed, PEG and PEG derivatives are common ingredients in personal care, beauty, and household cleaning products (e.g., soap, sunblock, cosmetics, detergent), as well as processed foods. Given the popular use of PEG in biologics and nanomedicines as well, the presence of pre-existing anti-PEG Ab poses significant concerns for the efficacy and safety of a wide range of therapeutics.

Despite these serious implications, the true prevalence and levels of pre-existing anti-PEG Ab responses remains not well understood. The reported prevalence of pre-existing anti-PEG Ab varies widely across different studies, ranging from <1% to 44%.[16,21,22] In addition to natural variations among subjects, the differences are likely due in part to the use of assay techniques such as hemagglutination tests or end point dilution ELISAs with different sensitivities and specificities of detection. Here, we sought to rigorously characterize pre-existing anti-PEG Ab in the general population by measuring the prevalence, concentration, and isotype of anti-PEG Ab in contemporary and historical plasma and serum samples from healthy adults. To enable reproducible quantitation of anti-PEG Ab that could serve as a reference for future investigations, we generated chimeric monoclonal anti-PEG IgG and IgM with known binding affinities to PEG and used them as standards in quantitative competitive ELISA assays.

EXPERIMENTAL SECTION

Human Plasma and Serum Samples.

Whole blood (K2- EDTA anticoagulant) from 68 individual healthy subjects was purchased from Bioreclamation (Hicksville, NY, USA), and the samples were centrifuged at 1500g for 15 min at room temperature. Aliquots of the top plasma layer were collected and stored at −80 °C until use. An 5additional 309 frozen plasma samples from healthy subjects were purchased from ProMedDx (Norton, MA, USA); samples were thawed, aliquoted, and stored at −20 °C until use. Serum collected during the periods 1970−1979, 1980−1989, and 1990−1999 (30, 30, and 19 samples, respectively) from patients with no history of malignancies were purchased from Mayo Clinic Bioservices (Rochester, MN, USA) and were thawed, aliquoted, and stored at −20 °C until use. Patient demographics are listed in Table 1.

Chimeric Anti-PEG Antibody Standards.

Sequences for chimeric anti-PEG Ab were generated by combining the V_H/V_L regions of commercially available murine anti-PEG Ab (6.3 IgG_1 and AGP.3 IgM; IBMS Academia Sinica)[23] with the C_H1/C_L and Fc regions of human IgG1−4 or IgM Ab. Plasmids encoding chimeric heavy and light chains along with J-chain (IgM only) were cotransfected into Expi293 cells (ThermoFisher) and grown for 72 h. The chimeric 6.3 (c6.3) IgG antibodies were purified from expression supernatant by single- step protein A/G purification (ThermoFisher) and assessed for purity by SDS-PAGE electrophoresis. Chimeric AGP.3 (cAGP.3) IgM antibodies were used directly from expression supernatant.

The concentration of the c6.3 IgG1−4 and cAGP.3 IgM standards were determined using ELISA. Briefly, high-binding half-area 96-well Costar plates (Corning) were coated with 500 ng/mL of antihuman Fab (#I5260, Sigma-Aldrich) or antihuman IgM (#609–1107, Rockland Immunochemicals) capture antibody in 50 nM bicarbonate buffer (pH 9.6) overnight at 4 °C. The chimeric antibodies were diluted in 1% milk in PBS-0.05% Tween and detected using antihuman IgM HRP (#609–1307, 1:75 000 dilution Rockland Immunochemicals), antihuman IgG HRP (#709–1317, 1:15 000 dilution, Rockland Immunochemicals), and/or corresponding IgG subclass secondary antibodies (see Anti-PEG Ab ELISA section). Pooled human IgG1 (Sigma-Aldrich), IgG2 (Abcam), IgG3 (Sigma-Aldrich), IgG4 (Sigma-Aldrich), and IgM (ImmunoReagents, Raleigh, NC, USA) were used as quantitation standards.

The K_D's of the generated chimeric Ab were determined using an Octet QK instrument (ForteBio, Menlo Park, CA, USA). Streptavidin biosensors were loaded with biotin-PEG_{10k}, and following a baseline step in DPBS with 0.01% bovine serum albumin (BSA), the sensors were then exposed c6.3 IgG1−4 or cAGP.3 at 0−100 nM in DPBS-0.01% BSA. Dissociation was monitored in DPBS-BSA. Raw data were processed using ForteBio's Data Analysis Software 6.4.

Anti-PEG Ab ELISA.

For detection of PEG-specific antibodies, 1,2-distearoyl-sn-glycero-3-phosphoethanolaminemethoxy PEG_{5k} (DSPE-PEG; Nanocs, New York, NY, USA) was coated onto medium-binding half-area 96-well Costar plates (Corning) at 50 μg/mL in DPBS overnight at 4 °C. After blocking the plates with 5% nonfat milk in DPBS, the plasma or serum samples, which were diluted 5- to 200-fold in 1% nonfat milk in DPBS, were added in the presence or absence of free diol-PEG_{8k} (4 mg/mL) and incubated for 1 h, followed by washes with DPBS. Antibodies bound to the DSPE-PEG coat were detected using the following detection antibodies and 1- step Ultra TMB (ThermoFisher): antihuman IgG_1 HRP (#A10648, 1:1000 dilution, ThermoFisher), mouse antihuman IgG_2 (#05–3500, 1:1000 dilution, ThermoFisher) along with antimouse IgG HRP secondary (#sc-2005, 1:4000 dilution, Santa Cruz Biotechnology), antihuman IgG_3 HRP (#053620, 1:1000 dilution, Invitrogen), antihuman IgG_4 HRP (#A10654, 1:750 dilution, ThermoFisher), or antihuman IgM HRP (#609–1307, 1:15000 dilution, Rockland Immunochemicals). After stopping the HRP reaction with 2 N sulfuric acid, the absorbance at 450 nm was measured using a Spectramax M2 plate reader (Molecular Devices). All wash and incubation steps

were performed using DPBS without any surfactant, as commonly used surfactants such as Tween contain PEG chains, which could artificially alter the ELISA results.[24] All assays included the respective c6.3 IgG1–IgG4 or cAGP.3 IgM standard curves (range 0−275 ng/mL), and the level of anti- PEG Ab present in the samples was determined using a 5-PL regression curve of the absorbance, which was corrected for the nonspecific background of sample wells treated with free diol- PEG_{8k}. Total anti-PEG IgG was calculated as the sum of the anti-PEG IgG1-IgG4 levels.

Detection cutoffs were established based on the corrected absorbance of the lowest standard curve point for each c6.3 IgG1−4 and cAGP.3 IgM standard curve according to the method described by Frey et al.[25] Assay precision was established by calculating the average coefficient of variation (CV%) for all detectable standard curve points, and the accuracy was calculated as 100% × observed concentration/ expected concentration for all detectable standard curve points (Supporting Information Table 1). To further confirm the specificity of the ELISA results, free methoxy-PEG_{40k}-myoglobin (PEG-MYO, Alfa Aesar) was used instead of free diol-PEG_{8k}, and the calculated anti-PEG IgG1, IgG2, and IgM concentrations obtained using the two different competitive molecules was compared for a range of samples.

Human Antibody Isotyping Quantification.

The levels of total IgG1, IgG2, IgG3, IgG4, and IgM in the human plasma and serum samples were quantified using a Bio-Plex Pro human isotyping 6-plex panel kit (BioRad) on a Luminex MAGPIX instrument (EMD Millipore) in duplicate according to the manufacturer's instructions. Samples were diluted 1:40 000 in the provided isotyping diluent. The total IgG1, IgG2, IgG3, IgG4, and IgM concentrations were calculated using MILLIPLEX Analyst 5.1 software, and the total IgG was calculated as the sum of the total IgG1−IgG4 levels.

Statistical Analyses.

Transformations for the anti-PEG IgM and IgG, as well as IgG1 and IgG2, variables were investigated using the Box-Cox procedure within generalized linear models. The covariates of interest were as follows: gender, age, age categorized, race, and historical time period (i.e, 1970−1979, 1980−1989, 1990−1999, and contemporary). The PEG IgM and IgG variables were also dichotomized to "above" and "below" their respective predetermined detection thresholds. Fisher's exact tests were used to evaluate general association for data categorized into contingency tables with nominal categories. Multivariable general linear models were also explored. Since this was an exploratory study, nominal (or unadjusted) p values have been reported. p values > 0.05 were considered significant. Statistical analyses were performed using SAS version 9.4 (SAS Institute), and all graphs were generated using Graphpad Prism version 6.0.

RESULTS AND DISCUSSION

Validation and Specificity of ELISA Assays for Measuring Anti-PEG Ab Levels.

Previous studies of anti- PEG Ab responses generally measured relative amounts of PEG-binding Ab through either hemagglutination assays with PEG-coated red blood cells or end point dilution ELISAs that determine Ab status based on absorbance readings above an often arbitrary threshold.[17,18,21,26–28] Unfortunately, both methods only estimate the relative amounts of Ab present, making comparisons between studies difficult. Quantitative ELISA offers the potential to provide precise measurements of absolute antibody concentrations but requires well-characterized Ab standards with a human Ab backbone in order to convert measured absorbance to absolute amounts of Ab. This led us to engineer chimeric monoclonal anti-PEG IgM and IgG1−4 based on merging human IgG1−4 and IgM backbones with PEG-binding V_H and V_L domains previously isolated from mouse IgM and IgG.[23] As expected, the four subclasses of chimeric anti-PEG IgG all possessed relatively similar binding affinities, with K_D values spanning the range of 4.8×10^{-9} to 5.8×10^{-9} M (Supporting Information Table 1). The K_D for chimeric anti-PEG IgM was 6.8×10^{-11}. One potential concern when using monoclonal Ab's as standards is the inherently polyclonal nature of endogenous serum Ab responses in individuals. Polyclonal Ab's can bind different portions of a pathogen or even different regions of a particular antigen and can therefore accumulate to a greater extent on the pathogen surface compared to a monoclonal Ab. Thus, monoclonal Ab's that bind only one specific epitope on an antigen are often not appropriate as antibody standards for characterizing a polyclonal response. However, since the PEG backbone consists of identical ethylene glycol repeats, the chimeric monoclonal Ab standards we developed must bind the same epitope (i.e., the repeating ethylene glycol units) as Ab originating from polyclonal anti-PEG Ab responses and thus can serve as appropriate Ab standards in quantitative ELISA assays.

A frequent criticism of prior anti-PEG ELISA measurements was the lack of confirmation of specificity to PEG. To ensure that we were indeed detecting PEG-specific Ab, we performed competitive binding with free diol-PEG_{8k}, and only reported the signal that was saturated by the free PEG. Using this competitive ELISA setup with chimeric Ab standards, our assay afforded sensitive detection of anti-PEG Ab, with final detection cutoff limits of 14.2 ng/mL, 15.1 ng/mL, 3.9 ng/mL, 4.4 ng/ mL, and 6.4 ng/mL for anti-PEG IgG1, IgG2, IgG3, IgG4, and IgM, respectively (Supporting Information Table 2). To further confirm specificity to PEG, we also tested an additional ELISA format using a methoxy-PEG_{40k}-myoglobin conjugate as the competing molecule instead of diol-PEG_{8k} and found comparable anti-PEG Ab levels for a range of samples (Supporting Information Figure 1).

In animals, anti-PEG Abs have been found against both the PEG backbone (CH_2-CH_2-O repeating units) and PEG terminal groups such as methoxy and hydroxy moieties.[24,29] Here, we chose to focus on backbone-specific anti-PEG Ab by using a methoxy-PEG_{5k}-lipid as the capture antigen and free diol-PEG_{8k} as the competing molecule in our competitive ELISA assay, for a number of reasons. First, the PEG backbone is by definition common to all PEGylated therapeutics, whereas terminal groups on different PEGylated systems can technically vary, even though all current PEGylated products currently on the market utilize

methoxy-terminal PEG. Second, many studies have reported anti-PEG Ab's that were specific to the PEG backbone and obtained identical results using PEG with different terminal groups. For example, Hershfield et al. found that binding of induced and pre-existing anti-PEG Ab was significantly inhibited by free diol-PEG_{10k}, leading the authors to conclude that anti-PEG Ab's detected were backbone- specific.[17] Similarly, Ganson et al. demonstrated that competition with free diol-PEG_{10k} and methoxy-PEG_{10k} reduced binding of pre-existing anti-PEG Ab to pegloticase to an equal extent.[22] Other existing literature reports of pre-existing and induced anti-PEG Ab's against other commercially available PEGylated drugs, including peginterferon beta-1a[30] and pegfilgrastim,[31] did not specify the terminal groups of the PEG molecules used to confirm the specificity of anti-PEG Ab's. Thus, while we can be certain that the reported Ab responses must encompass Ab's that bind the PEG backbone, the same may not be true of Ab's against PEG terminal groups.

Anti-PEG Ab Levels in the Contemporary Population.

Immune responses to therapeutic agents can reduce or completely eliminate their efficacy, as well as lead to undesirable side effects such as hypersensitivity and anaphylactic reactions that pose significant concerns for patient safety. Although PEG was long thought to be nonimmunogenic and had even been used to reduce the immunogenicity of protein antigens, growing evidence suggests that both animals and humans can form antibodies that specifically recognize the PEG component of various PEGylated therapeutics.[22,28,29] A particularly concerning phenomenon is the potential presence of anti-PEG Ab in treatment-naïve individuals without previous exposure to specific PEGylated therapeutics (i.e., individuals with pre-existing anti-PEG Ab).

To quantify the levels and prevalence of pre-existing PEG- specific Ab's in the general population, we screened a total of 377 commercially sourced plasma samples from healthy human blood donors for anti-PEG IgG and IgM levels using the competitive ELISA assay with diol-PEG_{8k} described above. Interestingly, we found that a high proportion of the plasma samples possessed detectable anti-PEG Ab levels. PEG-specific Ab levels statistically (90% confidence interval) above the detection cutoff limits were detected in ~72% of individuals, with 18%, 25%, and 30% of all samples possessing anti-PEG IgG only, anti-PEG IgM only, and both anti-PEG IgG and IgM, respectively (Table 2). Our findings differ substantially from previously reported prevalence rates for pre-existing anti-PEG Ab, which ranged from <1% to 44%,[16,32,33] with more recent studies averaging 20%−30% for healthy donors or treatment- naïve patients,[17,21,22,30] and which were generally determined using hemagglutination and end point dilution ELISAs. In our study, since most individuals exhibited only low anti-PEG Ab levels, the high prevalence of anti-PEG Ab is likely attributed in part to the high sensitivity of our competitive ELISA method (detection cutoff limits 2−15 ng/mL). Indeed, the majority of these "positive" plasma specimens had only low levels of anti- PEG Ab, with geometric mean anti-PEG IgG and IgM concentrations of 52 ng/mL and 22 ng/mL, respectively (Figure 1A,B). Using higher threshold values, ~37% of samples possessed anti-PEG Ab's above 100 ng/mL (28% IgG only, 6% IgM only, 3% both IgG and IgM), and plasma samples with anti-PEG IgG and/or IgM above 500 ng/mL represented only 8% of the total (Table 2), which would more closely align with the reported values in the existing literature.

Given the generally strong safety profile of many PEG- modified therapeutics, and assuming that the prevalence and concentrations of anti-PEG Ab measured here are correct, our findings would seem to support the conclusion that, under certain threshold concentrations, low to perhaps even modest levels of circulating anti-PEG Ab's in most individuals would not adversely affect the safety and efficacy of PEG-modified therapeutics. Consistent with this hypothesis, in several recent clinical trials, accelerated clearance or adverse reactions to PEGylated drug have primarily been reported in subjects with high titers of anti-PEG Ab.[19,22,28] Unfortunately, the precise threshold concentrations of anti-PEG Ab that could begin to impact the safety and efficacy of PEG-modified therapeutics remain poorly understood and likely vary depending on the specific therapeutic(s) in use. In clinical studies of monoclonal Ab drugs, low level antidrug Ab responses (100 ng/mL) have been associated with altered pharmacokinetics;[34,35] thus, the PK/PD of certain PEGylated proteins and drug delivery systems, as well as their safety profiles, may be sensitive to modest or even low levels of circulating anti-PEG Ab's. This possibility, coupled with the small but not trivial number of healthy individuals who exhibited high levels of pre-existing anti-PEG Ab (Table 2), suggests that sensitive detection and precise quantitation of anti-PEG Ab levels in a clinical setting will be essential to ensuring the safe use of PEGylated drugs in all target patient populations going forward.

The mechanism through which antibodies can be generated against a polymer that demonstrates strong antifouling properties remains a mystery. In rodent models, anti-PEG Ab formation has been proposed to occur through a T-cell independent mechanism that generates anti-PEG IgM almost exclusively and does not induce memory.[36–38] In contrast, we found a mixture of anti-PEG IgG and IgM in the human samples, with higher concentrations of anti-PEG IgG (Figure 1). The high presence of detectable anti-PEG IgG implies that immunological memory likely exists in the majority of the population that could in turn result in a rapid increase of anti- PEG Ab's following dosing of PEGylated drugs. When combined with the increasing use of PEGylation, this reality could present a unique medication management and polypharmacy issue, as patients may have elevated levels of pre-existing anti-PEG Ab's in response to an unrelated therapeutic prior to receiving the PEG-modified therapy of interest. Even the inclusion of PEG as an inactive ingredient could pose a challenge for individuals with sufficiently high anti- PEG Ab levels, as evidenced by the serious adverse reactions experienced by two patients in a clinical trial for PEGylated phenylalanine ammonia lyase who received unrelated intra- muscular injections of contraceptives containing PEG as an excipient.[19] Interestingly, Lubich et al. reported that repeated measurements of anti-PEG levels in some individuals can vary over time in the absence of known treatment with PEGylated drugs.[21] Further understanding of the mechanism, risk factors, critical threshold, and other characteristics of anti-PEG Ab would significantly improve our ability to identify, mitigate, or avoid PEG-related immunogenicity in patients.

Various IgG subclasses possess different effector functions and can potentially offer further insight into observed humoral immune responses, including the mechanism of Ab induction.[39] Thus, we evaluated the levels of different subclasses of anti- PEG IgG (IgG1−4) among the contemporary human plasma samples. Interestingly, detected anti-PEG IgG's were almost exclusively of the IgG1 and IgG2 subclasses, with IgG2 as the dominant subclass

(57% positive individuals, geometric mean 41 ng/mL) instead of IgG1 (26% positive individuals, geometric mean 12 ng/mL; Table 3, Figure 2). All 97 serum samples that were positive for anti-PEG IgG1 also contained anti-PEG IgG2. PEG-specific IgG3 and IgG4 were rarely detected (Table 3, Figure 2). In individuals positive for anti- PEG IgG, anti-PEG IgG1, IgG2, IgG3, and IgG4 accounted for ~16%, 83%, 0%, and 1% of the total detectable anti-PEG IgG on average. For comparison, the relative abundance of all IgG subclasses in humans is ~60%, 32%, 4%, and 4% for IgG1−4, respectively.[39] IgG2 antibodies are often associated with T-cell independent antibody induction, which would appear to support the mechanism of anti-PEG Ab induction observed in mice. The IgG2 subclass is primarily responsible for antibody responses to polysaccharide antigens;[39] PEG, as a highly repetitive and hydrophilic polymer, may bear some structural resemblance to such antigens. However, it should be noted that high levels of anti-PEG IgG2 alone do not necessarily signify a solely T-cell independent mechanism of antibody induction. The presence of anti-PEG IgG1, which was found in approximately half of all anti-PEG IgG2-positive individuals and comprised ~16% of the total anti-PEG IgG/individual on average, reflects a likely complex and variable mechanism of anti-PEG Ab formation.

We next performed linear regression analysis to evaluate the relationship between the prevalence and levels of pre-existing anti-PEG Ab and available demographics factors such as age, gender, and race. Both the concentration and prevalence of anti-PEG IgG, but not anti-PEG IgM, decreased with greater age ($p < 0.01$), with a 63% reduction in the prevalence between the oldest vs youngest age group (Figures 3A,B and 4). However, the extent of correlation between the anti-PEG IgG and age was weak, with $R^2 < 0.10$ for most analyses (Supporting Information Figure 2). The prevalence and serum levels of anti- PEG IgG were not correlated to gender, whereas females were slightly more likely to possess anti-PEG IgM ($p < 0.01$; Figure 3E,F). No observed relationship was found for race with anti- PEG IgM and IgG. Similar to total anti-PEG IgG, anti-PEG IgG1 and IgG2 concentrations were correlated with age (all $p < 0.01$) but not gender or race (Figure 3). To determine whether the observed relationship with age was attributed to a reduction in total antibody levels, we quantified the total concentrations of human IgG1−4 and IgM in all samples. The amount of anti- PEG IgG, IgG1, and IgG2 as a fraction of the total IgG, IgG1, and IgG2 levels, respectively, also decreased with age (all $p < 0.01$) but not with race or gender, indicating that the decline in prevalence and levels of anti-PEG Ab is not attributed to a broad decrease in total Ab. Whether the relationship between anti-PEG IgG and age reflects overall changes in the immune system with age (e.g., reduced B cell repertoire or reduced isotype switching from low affinity IgM to higher affinity IgG),[40] decreased affinity of the PEG-specific IgG in older vs younger individuals, differences in recent or total lifetime exposure to PEG, or other factors remains unclear at this time but may be partially revealed by longitudinal studies of anti- PEG Ab responses within the same individuals over time.

Anti-PEG Ab Levels in Historical Samples.

In the earliest report of human anti-PEG antibodies, Richter and Akerblom observed pre-existing anti-PEG Ab in <5% of healthy donors,[33] compared to later results such as those of Armstrong et al. in 2003 (27%–28% of healthy donors).[26] On the basis of these disparities, it has been hypothesized that anti-PEG immunity may be gaining in prevalence, with the

increased use of PEG in household, food, beauty, and health products being a primary driver of the change. To determine whether these differences reflect a true increase in anti-PEG Ab levels among the population over time or are likely attributed to differences in sensitivity of detection assays, we obtained healthy human serum samples banked from the 1970s, 1980s, and 1990s and quantified the levels of anti-PEG Ab in these samples. We detected anti-PEG IgG alone, anti-PEG IgM alone, and both anti-PEG IgG and IgM in 20%, 19%, and 16% of all historical samples, respectively (Table 4, Figure 5). Although the overall prevalence of anti-PEG Ab among the historical samples was slightly lower than among the contemporary samples ($p < 0.001$), the observed prevalence rates of anti-PEG Ab were far higher than those previously reported in historical human samples (e.g., 0.2%−4.9% in healthy donors in 1984[33]). In contrast, the measured concentrations of anti-PEG IgG and IgM did not differ significantly between the historical and contemporary samples. Other major characteristics of the anti- PEG Ab response, such as higher concentrations of IgG relative to IgM and the presence of IgG2 as the dominant anti-PEG IgG subclass, were consistent between historical and contemporary samples as well (Figure 5, Supporting Information Figures 2 and 3). The amount of total antibody present in the historical serum samples was similar to that in the contemporary plasma samples (Supporting Information Figures 5), indicating that the obtained samples remained intact over years of storage and were unlikely to have negatively impacted the measured anti-PEG Ab lssevels. We observed no clear relationship between any demographic factors and anti-PEG Ab prevalence or concentration for the historical samples, although the small sample size likely limited the power of our analysis. Overall, given that over 50% of the serum samples from the 1970s to 1990s actually possessed anti-PEG Ab, our results suggest that the hypothesis of emerging anti-PEG prevalence may not be true. Instead, immunological responses to PEG are likely longstanding yet underappreciated, and the increasing incidence of adverse events with PEG-modified therapeutics may simply reflect its increasing parenteral use in pharmaceutical and clinical settings.

CONCLUSIONS

PEG has a variety of useful applications in the pharmaceutical industry, and a number of PEGylated therapeutics have been highly successful. However, growing evidence from recent clinical trials suggests that the presence of high anti-PEG Ab levels, including pre-existing humoral responses, can abrogate the efficacy of PEG-modified drugs or result in serious adverse reactions. Using a rigorously validated quantitative ELISA method, we detected low levels of anti-PEG IgG and IgM in the majority of the population and high levels (>500 ng/mL) in a small but nontrivial number of individuals. The presence of anti-PEG implies the existence of immunological memory that could result in rapid elevation of anti-PEG Ab levels. In light of the increasing number of PEG-modified or PEG-containing pharmaceutical products on the market, we believe it is prudent to introduce regular monitoring of anti-PEG Ab responses in patients receiving PEGylated therapies, as it could affect clinical trial design, testing, and dosing regimens for PEGylated therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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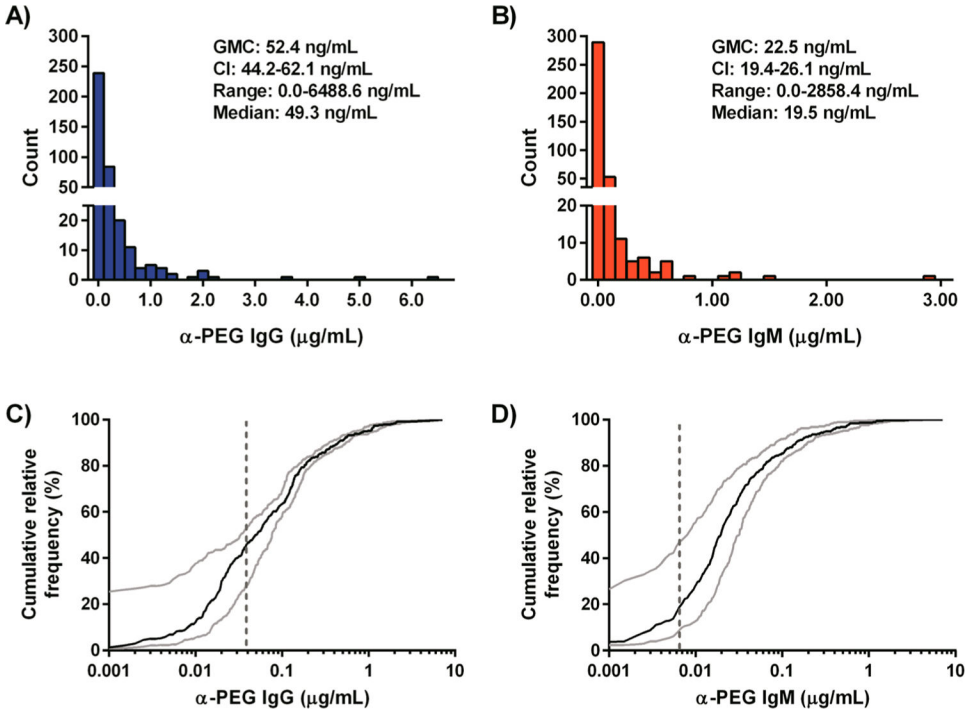

Figure 1.

Frequency distribution of (A) anti-PEG IgG and (B) anti-PEG IgM levels in contemporary human plasma samples (n = 377). GMC, geometric mean concentration; CI, 95% confidence intervals for the GMC. Cumulative frequency distribution of (C) anti-PEG IgG and (D) anti-PEG IgM levels in contemporary human plasma samples. Light gray lines represent the 90% CI, and detection cutoff limits are indicated by the vertical gray dashed lines.

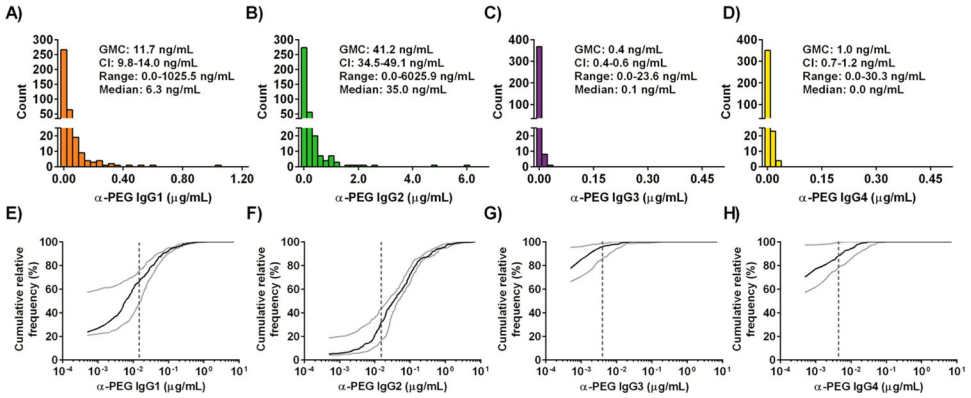

Figure 2.

Frequency distribution of (A) anti-PEG IgG1, (B) IgG2, (C) IgG3, and (D) IgG4 levels in contemporary human plasma samples (n = 377). GMC, geometric mean concentration; CI, 95% confidence intervals for the GMC. Cumulative frequency distribution of (E) anti-PEG IgG1, (F) IgG2, (G) IgG3, and (H) IgG4 levels in contemporary human plasma samples. Light gray lines represent the 90% CI, and detection cutoff limits are indicated by the vertical gray dashed lines.

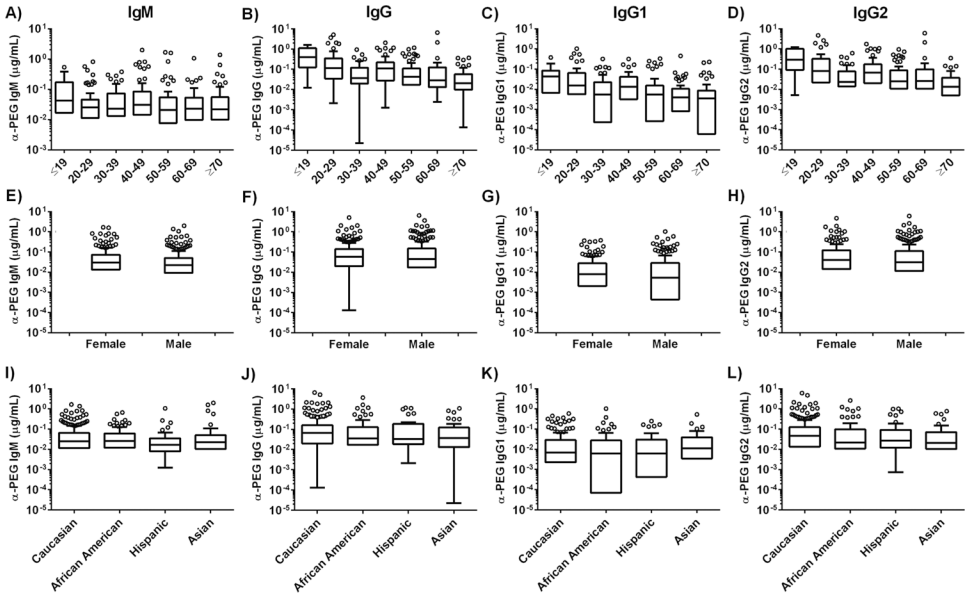

Figure 3.

Anti-PEG IgM, IgG, IgG1, and IgG2 levels by (A–D) age group, (E–H) gender, and (I–L) race in healthy individuals (n = 377). The data are depicted using Tukey's method for box-and-whisker plots, with samples outside of the whiskers shown as open circles.

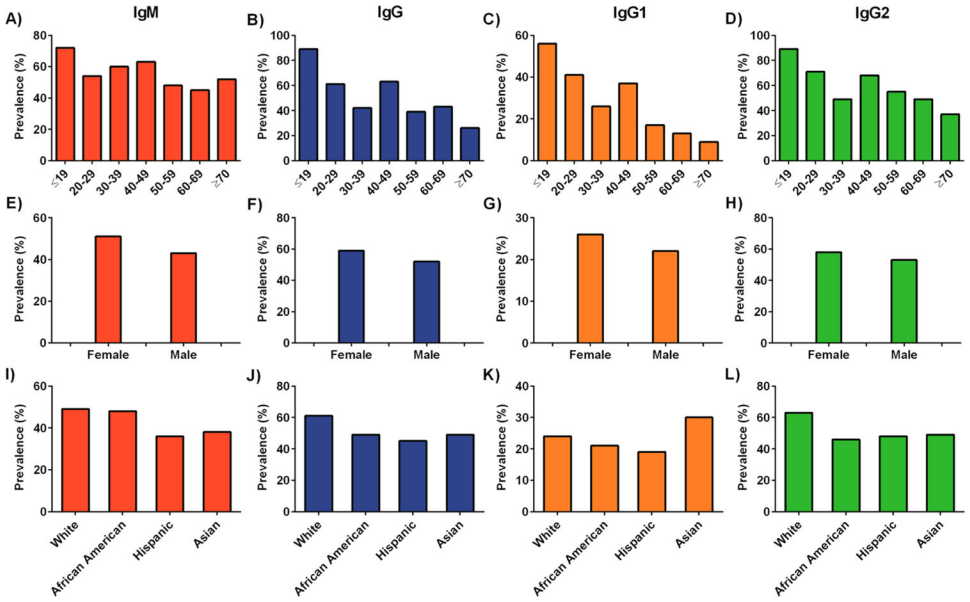

Figure 4.

Anti-PEG IgM, IgG, IgG1, and IgG2 prevalence by (A–D) age group, (E–H) gender, and (I–L) race in healthy individuals (n = 377).

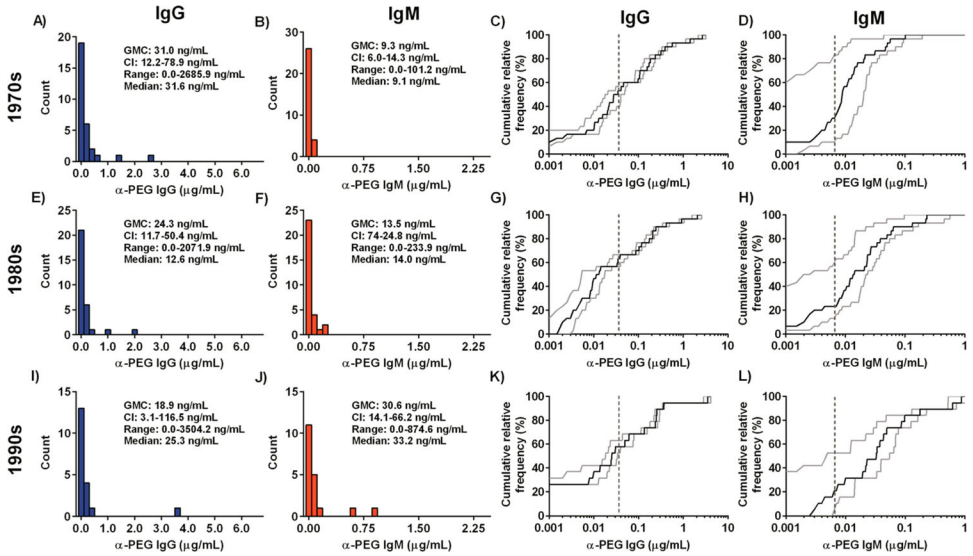

Figure 5.

Frequency distribution of anti-PEG IgG and IgM levels in historical human serum samples collected in the (A,B) 1970s, (E,F) 1980s, and (I,J) 1990s (n = 30, 30, and 19, respectively). GMC, geometric mean concentration; CI, 95% confidence intervals for the GMC. Cumulative frequency distribution of anti-PEG IgG and IgM levels in historical human serum samples collected in the (C,D) 1970s, (G,H) 1980s, and (K,L) 1990s. Light gray lines represent the 90% CI, and detection cutoff limits are indicated by the vertical gray dashed lines.

Table 1.

Summary of Patient Demographics for Contemporary and Historical Samples

	contemporary (*n* = 377)	**1970–1979 (*n* = 30)**	**1980–1989 (*n* = 30)**	**1990–1999 (*n* = 19)**
age, n (%)				
≤ 19	18 (5%)	0 (0%)	1 (3%)	0 (0%)
20–29	56 (15%)	8 (27%)	5 (17%)	0 (0%)
30–39	65 (17%)	7 (23%)	5 (17%)	0 (0%)
40–49	62 (16%)	8 (27%)	5 (17%)	9 (47%)
50–59	69 (18%)	7 (23%)	5 (17%)	5 (26%)
60–69	53 (14%)	0 (0%)	5 (17%)	4 (21%)
≥ 70	54 (14%)	0 (0%)	4 (13%)	1 (5%)
gender, *n* (%)				
Male	226 (60%)	15 (50%)	15 (50%)	13 (68%)
Female	151 (40%)	15 (50%)	15 (50%)	6 (32%)
race, *n* (%)				
Caucasian	200 (53%)	30 (100%)	30 (100%)	19 (100%)
Black/African American	49 (13%)	0 (%)	0 (%)	0 (%)
Hispanic	42 (11%)	0 (%)	0 (%)	0 (%)
Asian	37 (10%)	0 (%)	0 (%)	0 (%)

Table 2.

Prevalence of anti-PEG IgG and IgM in Contemporary Human Plasma Samples (n = 377)

prevalence of anti-PEG Ab response	total Ab	IgG	IgM	IgG and IgM
positive individuals, n (%)	273 (72%)	67 (18%)	93 (25%)	113 (30%)
individuals ≥ 100 ng/mL, n (%)	139 (37%)	107 (28%)	22 (6%)	10 (3%)
individuals ≥ 500 ng/mL, n (%)	30 (8%)	26 (7%)	4 (1%)	0 (0%)

Table 3.

Prevalence of Anti-PEG IgG1–4 in Contemporary Human Plasma Samples (n = 377)

prevalence of anti-PEG Ab response	IgG1	IgG2	IgG3	IgG4
positive individuals, n (%)	97 (26%)	214 (57%)	5 (1%)	3 (1%)
individuals ≥ 100 ng/mL, n (%)	19 (5%)	83 (22%)	0 (0%)	0 (0%)
individuals ≥ 500 ng/mL, n (%)	1 (0%)	19 (5%)	0 (0%)	0 (0%)

Table 4.

Prevalence of Anti-PEG IgG and IgM in Historical Human Serum Samples Collected from the 1970s, 1980s, and 1990s (n = 30, 30, 19, Respectively)

	prevalence of anti-PEG Ab response	total Ab	IgG	IgM	IgG and IgM
1970s	positive individuals, n (%)	14 (47%)	8 (27%)	2 (7%)	4 (13%)
	individuals ≥ 100 ng/mL, n (%)	9 (30%)	9 (30%)	0 (0%)	0 (0%)
	individuals ≥ 500 ng/mL, n (%)	2 (7%)	2 (7%)	0 (0%)	0 (0%)
1980s	positive individuals, n (%)	18 (60%)	5 (17%)	8 (27%)	5 (17%)
	individuals ≥ 100 ng/mL, n (%)	7 (23%)	7 (23%)	0 (0%)	0 (0%)
	individuals ≥ 500 ng/mL, n (%)	2 (7%)	2 (7%)	0 (0%)	0 (0%)
1990s	positive individuals, n (%)	12 (63%)	3 (16%)	5 (26%)	4 (21%)
	individuals ≥ 100 ng/mL, n (%)	8 (42%)	5 (26%)	2 (11%)	1 (5%)
	individuals ≥ 500 ng/mL, n (%)	3 (16%)	1 (5%)	2 (11%)	0 (0%)