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Submicron Size Particles of a Murine Monoclonal Antibody Are More Immunogenic Than Soluble Oligomers or Micron Size Particles Upon Subcutaneous Administration in Mice

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ABSTRACT

Protein aggregates are one of the several risk factors for undesired immunogenicity of biopharmaceuticals. However, it remains unclear which features determine whether aggregates will trigger an unwanted immune response. The aim of this study was to determine the effect of aggregates' size on their relative immunogenicity. A monoclonal murine IgG1 was stressed by exposure to low pH and elevated temperature followed by stirring to obtain aggregates widely differing in size. Aggregate fractions enriched in soluble oligomers, submicron size particles and micron size particles were isolated via centrifugation or size-exclusion chromatography and characterized physicochemically. The secondary and tertiary structures of aggregates were altered in a similar way for all the fractions, while no substantial chemical degradation was observed. Development of anti-drug antibodies was measured after subcutaneous administration of each enriched fraction to BALB/c mice. Among all tested fractions, the most immunogenic was the one highly enriched in submicron size particles (~100-1000 nm). Fractions composed of micron size (>1-100 μm) particles or soluble oligomers (<100 nm) were not immunogenic under the dosing regimen studied in this work. These results show that aggregate size is an important factor for protein immunogenicity.

Introduction

Monoclonal antibodies are the most widely used class of therapeutic proteins.[1] Reported rates of anti-drug antibodies (ADA) formation in patients during mAb treatments vary from 0% to almost 90%.[2,3] ADA generation can lead to a decrease in efficacy by altering the pharmacokinetics and pharmacodynamics, potentially resulting in severe clinical consequences.[2-5] The presence of protein aggregates in formulated drug is commonly believed to be one of the crucial risk factors contributing to ADA formation. A number of *in vitro* and *in vivo* studies have confirmed that aggregated proteins are more immunogenic than monomeric ones.[6-11] Moreover, it has been suggested that aggregates with different features significantly differ in their ability to trigger ADA.[11,12] Unfortunately, in the majority of studies, unfractionated protein aggregates were tested for their ability to trigger ADA, so it remains unclear which attributes contribute to the immunogenicity of protein aggregates.

Size is one of the most commonly used ways in which aggregates are classified. However, data linking aggregate size to immunogenicity is very limited. Only a few preclinical reports are available in which immunogenicity of aggregates was studied *in vitro* or *in vivo* with respect to their size.[13-16] Moreover, the results of these studies are somewhat conflicting. In part of them, micron-sized aggregates were found to be the more immunogenic than other tested sizes.[15,16] Other studies correlated higher immunogenicity with smaller aggregates, for example, oligomers.[14] However, these studies often included only aggregates of a narrow size range and structurally different aggregates. For example, Bessa et al.[13] showed that oligomers are more immunogenic in mice than monomers, but they did not study larger aggregates. In another report, Fathallah et al.[14] suggested higher immunogenicity of

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oligomeric aggregates (10-40 nm) than micron size particles. However, the structure of proteins forming micron size particles was highly disrupted in contrast to native-like protein molecules found in oligomers. Several studies have shown that aggregates composed of fully denatured protein molecules do not elicit antibodies cross-reacting with the native species, in contrast to aggregates of proteins with considerable native-like structure.[17] Another factor blurring the available data is the fact that in current literature multiple distinct aggregate size classifications can be found. Some of them are based on subjective terms, for example, "subvisible particles" or "soluble oligomers" and not on measurable features like diameter or volume.[18] Therefore, currently there is no consensus on the role of aggregate size in triggering of ADA.

The aim of our study was to compare the relative immunogenicity of mAb aggregates in a wide size range (10 nm-100 μm) upon subcutaneous (SC) administration in Balb/c mice. We have chosen this mouse strain because it has been widely used in immunogenicity studies, either directly as "wild type" model, as background strain for the development of transgenic mouse models, or cross-bred with other mouse strains to obtain the desired model.[15,19-21] To avoid introduction of highly immunogenic foreign epitopes, which might mask the effect of aggregation on immunogenicity, recombinant monoclonal murine IgG1 (mIgG1) was used as a model protein. Such an approach has been previously used to mimic a patient's immune tolerance status toward recombinant human therapeutics.[17,22] The IgG1 was subjected to stress conditions to generate aggregates of various sizes with similar structural features. The obtained aggregates were separated according to their size by either centrifugation or size-exclusion chromatography (SEC). Fractions enriched in different sizes of aggregates were then injected SC into mice, and immunogenicity was determined by measuring ADA levels in serum. The fraction enriched in submicron size particles was found to be significantly more immunogenic than fractions containing mainly micron size particles or soluble oligomers smaller than 100 nm.

Materials and Methods

Monoclonal Antibody

Recombinant monoclonal mIgG1 (pI: 6.8) targeting an *E coli* pilus FimH was provided by MedImmune. The mIgG1 was formulated in histidine buffered saline (25 mM histidine, 150 mM NaCl, pH 6.0). Before stress treatment, the mIgG1 was buffer-exchanged into phosphate buffered saline (PBS, 1.54 mM KH_2PO_4, 2.7 mM Na_2HPO_4-7 H_2O, 155 mM NaCl, pH 7.2) obtained from Life Technologies (Paisley, UK). PD-10 columns (GE Healthcare, Buckinghamshire, UK) were used according to the manufacturer's protocol to replace the formulation buffer.

Generation and Fractionation of Aggregates

Aggregation was generated according to the protocol developed for a fluorescently labeled version of the same mIgG1 and described by Kijanka et al.[23] However, as unconjugated mIgG1 displayed a slightly better stability than labeled mIgG1, a higher temperature (59°C instead of 55°C) and a longer incubation time (63 min vs. 60 min) were used to generate aggregates (see Table 1 for overview of the prepared solutions). In brief, for centrifugation-based fractionation, the mIgG1 was diluted in PBS to a final concentration of 2 mg/mL. Next, 500 μL of mIgG1 solution was mixed with an equal volume of "low pH buffer" (0.1 M Na_2HPO_4, 0.05 M citric acid, pH 4.6). The obtained solution was first incubated at 59°C for 63 min, followed by 30 min of stirring (700 rpm) at room temperature. After neutralization with 1 M NaOH, the solution was centrifuged (3000 × *g*, 10 min, 4°C). "Supernatant," the fraction enriched in submicron size particles, was transferred into a new tube. "Pellet," the fraction enriched in micron size particles, was suspended in fresh PBS by pipetting "up-and-down" 10 times.

Table 1
Overview of the Samples Prepared and Studied in This Article

Sample	Stress Treatment	Isolation
"Unstressed"	-	-
"SEC monomers"	pH 4.6, 59°C, 30 min	SEC
"SEC oligomers"	pH 4.6, 59°C, 30 min	SEC
"SEC oligomers purified"	pH 4.6, 59°C, 30 min	SEC + centrifugation (18,000 × *g*, 30 min)
"SEC unfractionated"	pH 4.6, 59°C, 30 min	-
"Supernatant"	pH 4.6, 59°C, 63 min + stirring, 30 min	Centrifugation (3000 x g, 10 min)
"Pellet"	pH 4.6, 59°C, 63 min + stirring, 30 min	Centrifugation (3000 x g, 10 min)

For SEC-based fractionation, 500 μL of mIgG1 (14 mg/mL in PBS) was mixed with an equal volume of "low pH buffer." Next, the solution was incubated at 59°C for 30 min. The sample was cooled down at 4°C for 10 min and neutralized with 1 M NaOH. This stressed, unfractionated sample is referred to as "SEC Unfractionated." The resulting solution was filtered through a 0.22 μm filter (Millipore, Carrigtwohill, Ireland) and fractions were isolated via SEC on an Agilent 1200 system (Agilent Technologies, Palo Alto, CA) equipped with an autoinjector and fraction collector. Fractions were separated on a High Load Superdex 200 PG column (GE Healthcare). A volume of 0.9 mL of aggregated protein solution was injected onto the column, PBS was used as mobile phase, and the flow rate was 1 mL/min. The fraction collected between 45-50 min of separation, referred to as "SEC Oligomers," was enriched in oligomers and submicron size particles (see Supplementary Fig. S1). Next, 2.5 mL of "SEC Oligomers" fraction was further purified by filtration through a 0.1-μm polyvinylidene fluoride filter (Millipore) followed by centrifugation (18,000 × *g*, 30 min, 4°C). The supernatant thus obtained, referred to as "SEC Oligomers Purified," was transferred into a new tube. The monomers subjected to stress conditions were collected between 65-70 min of separation and are referred to as "SEC monomers." All samples were prepared freshly at the day of administration and were stored for up to 4 h at 4°C prior to injection.

The total protein concentration of all aggregated species in the obtained fractions was determined by microBCA assay (Thermo Fisher Scientific, Rockford, IL) according to the supplier's protocol. Samples were screened for endotoxin contamination with the Pierce® LAL Chromogenic Endotoxin Quantitation Kit according to the manufacturer's protocol (Thermo Fisher Scientific). For all tested fractions, except "GPC Monomer," the measured endotoxin level was below the detection limit of the assay, that is, <0.1 endotoxin unit (EU) per mL of the injected solution or <0.02 EU/injected dose. The endotoxin levels in "GPC Monomer" were at maximum 0.5 UE/mL, corresponding to 0.1 EU/injected dose. As an adult Balb/c mouse weighs about 25 g, the maximum administered dose of endotoxins in "GPC Monomer" was 4 EU/kg/h, which is below the United States Pharmacopeia <85 > chapter's limit for injectable solutions of 5 EU/kg/h[24]

Characterization of Aggregates

High-Performance SEC

High-performance (HP-SEC) was used to quantify the content of monomers and oligomers in isolated fractions. Samples were analyzed on an Agilent 1200 system (Agilent Technologies)

equipped with an autoinjector, an absorbance detector, and a multiangle laser light scattering (MALLS) Dawn Helios detector (Wyatt Technology, Dernbach, Germany). Before analysis, all samples were centrifuged (10,000 × *g*, 10 min, 4°C). Fifty micrograms of each fraction were analyzed on a TSK Gel 4000 SWXL column (Tosoh Bioscience, King of Prussia, PA). PBS supplemented with 0.2 M arginine was used as the mobile phase and the flow rate was 1 mL/min. The protein peaks were detected at 280 nm, and the molecular weight of the eluting material was calculated on the basis of MALLS data in Astra V 5.3.4.20 software (Wyatt Technology).

Dynamic Light Scattering

Samples were analyzed with a Malvern Zetasizer Nano ZS (Malvern, Herrenberg, Germany) equipped with a 633 nm He-Ne laser operating at an angle of 173°. Data were collected with the Zetasizer Software v.7.11 (Malvern). A 500 μL of each fraction was measured in a polystyrene semi-micro cuvette (Brand, Wertheim, Germany) with a 10 mm path length. The measurements were performed at 25°C with an automatic attenuation, run duration, and number of runs. The mean Z-average (Z_{ave}) diameter and polydispersity index (PDI) are reported.

Nanoparticle Track Analysis

Nanoparticle track analysis (NTA) was used to determine the number of submicron size particles (size range 0.1-1 μm) in the samples. The measurements were performed with a NanoSight LM20 (NanoSight, Amesbury, UK), equipped with a sample chamber with a 640 nm laser and a syringe pump. The samples were measured for 90 s with manual adjustment of shutter and gain. Each sample was measured 3 times. The "Unstressed," "SEC monomers," "SEC oligomers," "SEC Oligomers Purified," "SEC Unfractionated," and "Pellet" fractions were diluted 10-fold with PBS prior to analysis, whereas "Supernatant" needed to be diluted 100-fold because of the very high particle load. The data were acquired and analyzed by the NTA 2.3 software (NanoSight).

Micro-Flow Imaging

The number of micron size aggregates (size range 1-100 μm) was determined by an micro-flow imaging (MFI) DPA4100 series A system (ProteinSimple, Santa Clara, CA) equipped with a silane coated 100 μm flow cell (ProteinSimple) and operated at high magnification (14×). Samples of 0.55 mL with a prerun volume of 0.2 mL were analyzed at a flow rate of 0.17 mL/min ($n = 3$). The "Unstressed," "SEC monomers," "SEC oligomers," "SEC Oligomers Purified," and "Supernatant" fractions were diluted 10-fold with PBS prior to analysis. For analysis of "Pellet" and "SEC unfractionated" samples, a 100-fold dilution was necessary. Data were acquired by MFI View software, version 6.9 and analyzed with MVAS, version 1.2 (ProteinSimple).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

One microgram of protein from each fraction was separated on 4%-15% Mini-Protean® TGX™ precast gel (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). A Laemmli sample buffer (Bio-Rad Laboratories B.V.) was used for samples analyzed under nonreducing conditions. To obtain reducing conditions, β-mercaptoethanol (Bio-Rad Laboratories B.V.) was added to Laemmli buffer to a final concentration of 355 mM. Prior to loading on the gel, samples were boiled at 95°C for 5 min. The electrophoresis was performed with a Biorad Mini-Protean module and 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 (Bio-Rad Laboratories B.V.) as a running buffer. Separation was initiated with electrophoresis at 80 V for 10 min, followed by 120 V for 50 min. Spectra™ Multicolor High Range Protein Ladder (Thermo Fisher Scientific) and Precision Plus Protein™ All Blue Standards (Bio-Rad Laboratories B.V.) were included for molecular weight determination. Silver Stain Plus kit (Bio-Rad Laboratories B.V.) was used for visualization of the protein bands. The images of gels were acquired with a GS-900 densitometer (Bio-Rad Laboratories B.V.) and Image Lab v.5.2.1 software (Bio-Rad Laboratories B.V.).

Western Blotting and Dot Blotting

For Western blot analysis, 0.1 μg protein of each fraction was separated by SDS-PAGE under nonreducing conditions as previously described. SDS-PAGE gels were blotted onto a supported nitrocellulose sheet (Bio-Rad Laboratories B.V.) with a Biorad Mini Trans-Blot electrophoretic transfer cell. Running buffer was composed of 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3. After blotting, the membranes were blocked overnight at 4°C with 2% bovine serum albumin (BSA) in PBS supplemented with 0.05% Tween 20 (PBST). Next, the blots were incubated for 1 h at ambient temperature with peroxidase labeled anti-mouse total IgG, IgG1, IgG2a (Southern Biotech, Birmingham, AL), all diluted 2000-fold in PBST. Then, the membranes were washed 3 times with PBST followed by 2 PBS washes and developed with AEC chromogenic substrate (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) according to the manufacturer's protocol.

In addition to blots developed with commercially available detecting mAb, mIgG1 aggregates were visualized with specific anti-mIgG1 polyclonal antibody purified from sera of rabbits immunized with mIgG1 by Covance (Denver, PA). After blocking, the membrane was incubated for 1 h at ambient temperature with rabbit anti-mIgG1 diluted 2000-fold in PBST. Next blot was first washed 3 times with PBST and then incubated for another 1 h with peroxidase-labeled anti-rabbit Ab (Thermo Fisher Scientific), diluted 2000-fold in PBST. Finally, the membrane was washed and developed with AEC chromogenic substrate as previously described.

Dot blots were prepared by applying of 0.1 μg of each fraction on a nitrocellulose membrane. After air drying of the samples, blots were blocked and developed according to protocols used for Western blots.

Far-UV Circular Dichroism Spectroscopy

For far-UV circular dichroism (CD), the mIgG1 fractions were diluted in PBS to 0.1 mg/mL. The CD spectra were measured with a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan) in a quartz cuvette with a path length of 0.2 cm at 25°C. The CD spectra were collected from 200 to 260 nm at a speed of 20 nm/min, a data pitch of 0.5 nm, a response time of 8 s, and a bandwidth of 1 nm. Five accumulations were collected for each sample. Spectra were smoothed with GraphPad Prism®, v 5.02 (GraphPad Software, Inc., La Jolla) by using 0^{th} order polynomial smoothing and 4 neighbors on each value as described previously.[25] Next, spectra were background corrected for the baseline spectrum of PBS. A mean residue ellipticity ([θ] mean residue weight [MRW]) was calculated according to Kelly et al.,[26] using an MRW of 112.35 (MRW = M/N-1, where M is the molecular mass, i.e., 148,300 Da, and N is number of amino acids residues of mIgG1, [i.e., 1320]).

Fluorescence Spectroscopy

The fluorescence emission spectra of isolated fractions were measured with a steady state fluorimeter FS900 (Edinburgh Instruments Ltd., Livingston, UK). All measurements were performed in a quartz cuvette of 1 cm path length and 1 mL of 0.1 mg/mL sample was used. The intrinsic fluorescence of tryptophan residues was recorded from 310 to 400 nm on excitation at 295 nm using slits of 3 nm, a dwell time of 1 s, steps of 0.5 nm, and a cumulative addition of 3 scans for each spectrum. All spectra were first

smoothed as previously described and next background corrected for the spectrum of the PBS.

Next, 4,4'-dianilino-1,1'-binaphthyl-5,5′-disulfonic acid dipotassium salt (Bis-ANS; Sigma-Aldrich B.V.) was added to each fraction to a final concentration of 1 μM. The Bis-ANS was excited at 385 nm, and the fluorescence emission was recorded from 410 nm to 600 nm with 1 nm steps and a dwell time of 1 s. Both excitation and emission slits were set to 5 nm, and a cumulative addition of 3 scans for each spectrum was collected. Obtained spectra were smoothed and buffer corrected as previously described.

Tryptic Peptide Mapping Using Liquid Chromatography Coupled With Mass Spectrometry

Assessment of potential posttranslational modifications (PTMs) and stress-induced chemical degradation was performed by tryptic digest peptide mapping of unstressed mIgG and unfractionated aggregated mIgG samples. Denaturation and reduction of 100 μg protein (100 μL protein samples at 1 μg/μL) was performed by addition of 200 μL of 8 M guanidine, 130 mM Tris, 1 mM EDTA, pH 7.6, and 10 μL of 500 mM DTT (Thermo Fisher Scientific, Waltham, MA), followed by incubation for 30 min at 37°C. Samples were alkylated with the addition of 25 μL of 500 mM iodoacetamide (G-Biosciences, St. Louis, MO) followed by a 30 min incubation while protected from light at ambient temperature. Samples were buffer-exchanged into 2 M urea, 100 mM Tris, pH 7.6 by centrifugal filtration with a 10 kDa MW membrane (Millipore Sigma, St. Louis, MO). Samples were digested at 37°C for 4 h by using 5 μg trypsin, then quenched with 4% trifluoroacetic acid and collected for analysis.

The digested peptides were separated on a Waters Acquity UPLC system with autosampler and tunable ultraviolet detector (Waters Corporation, Milford, MA) equipped with a BEH300 C18 column (1.7 μm, 2.1 × 150 mm) (Waters Corporation) using a gradient elution from 100% to 65% mobile phase A (0.02% TFA in water, mobile phase B was 0.02% TFA/acetonitrile) at a flow rate of 0.2 mL/min (total elution time of 78 min). Eluted peptides were detected by UV absorbance at 220 nm and analyzed by a Waters Synapt G2 QTOF mass spectrometer (Waters Corporation). Data were analyzed using Waters UNIFI and MassLynx software (Waters Corporation).

Estimation of Protein Mass Within Different Size Ranges of Aggregates

HP-SEC, NTA, and MFI were used to calculate the mass of protein in different size ranges for each enriched fraction. The total estimate of protein mass in the different size ranges for each of the injected fractions was compared to the experimental microBCA assay value for total protein mass in the fractions to assess the accuracy of the calculated compositions. The mass of mIgG1 fragments, monomers, and oligomers was calculated from the peak areas under the curve (AUC) in the HP-SEC analysis. The "Unstressed" controls were used as references for recovery calculations. The AUC of unstressed mIgG1 (100%) corresponded to the mass of analyzed protein, that is, 50 μg. The recovery of stressed fractions was calculated by comparing their AUC to that of the corresponding "Unstressed" mIgG1.

The mass of protein in submicron size particles and micron size particles was calculated on the basis of NTA and MFI data as described previously.[23,27,28] In short, mass was assessed according to the formula proposed by Barnard et al.:[27] $M = d*V*n*p$, where d is the density of the protein (1.4 mg/mL), V is the volume of particles per size bin (width: 1 nm for NTA and 0.25 μm MFI), n is the number of particles per size bin, and p is the fraction of the particle volume (V) occupied by protein (assumed to be 0.75). The mass of all submicron size particles was obtained by summing up the masses of the particles in all size bins determined by NTA. The same strategy was used to estimate the mass of micron size particles determined by MFI. The calculated mass composition of fractions is expressed as percentage of total protein per mL measured with microBCA assay.

Animal Study

Mice

BALB/c mice of 6-8 weeks age were obtained from Charles River Laboratory (L'Arbreske Cedex, France) and kept in standard cages with access to food and water (acidified) *ad libitum*. All testing was conducted with approval of the Animal Ethic Committee of Leiden University Medical Center (permission number 14096).

Animal Experiments

In the first experiment, referred to as experiment I, a total number of 84 mice was divided into 7 groups ($n = 12$) of which 6 were treated with different stressed fractions and one with unstressed mIgG1 as a control. Mice were injected SC between the shoulders twice per week for 8 weeks with 10 μg of protein per injection diluted in endotoxin free PBS. BD Micro-Fine™ 0.5 mL insulin syringes with a permanently attached 31G needle (Becton Dickinson B.V., Breda, The Netherlands) were used for the injections. The basal level of ADA was determined for each mouse in blood collected submandibularly prior to the first injection. From each mouse, blood was collected every second week. Mice were sacrificed 2 weeks after the last injection (day 65). The administration regimen is schematically shown in Figure 1. This study design, consisting of 16 injections of 10 μg of protein per injection over an 8-week time period, has been chosen based on pilot studies and was found to be the most discriminative among the tested regimes.

In the follow-up experiment, referred to as experiment II, mice ($n = 16$ per group) were administered with 3, 10, and 30 μg of "Supernatant" fraction per injection to determine the impact of aggregates' dose on the immunogenicity. Unstressed mIgG1 was used as a control ($n = 16$). Administration route and duration, injection frequency, and blood collection regimen described previously were used. One week after the first 2 injections (day 10), 4 mice from each group were euthanized, and spleens as well as draining lymph nodes (LNs), that is, brachial and axillary LNs, were extracted. The remaining mice were sacrificed on day 65.

Blood was collected into the MiniCollect® Serum Z separator tubes (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Serum was isolated by centrifugation (3000 × *g*, 10 min), collected into storage tubes (Thermo Fisher Scientific) and kept at −80°C for analysis.

Anti-drug Antibody Detection

The ADA screening was performed with a bridging ELISA according to an adopted protocol previously described by Qiu et al.[29] The capture and detection reagents, mIgG1-biotin and mIgG1-digoxigenin, were obtained by labeling of mIgG1 with EZ-Link® sulfo-NHS-LC-biotin (Thermo Fisher Scientific) or 3-amino-3-deoxydigoxigenin hemisuccinamide, succinimidyl ester (Thermo Fisher Scientific) according to the manufacturer's protocols. Dulbecco's PBS (Life Technologies) supplemented with 0.5% BSA (Sigma-Aldrich Chemie B.V) was used as the assay buffer. The polyclonal rabbit anti-mIgG1 antibody used as the positive control in ADA assay was generated and purified by Covance. Samples and polyclonal ADA controls (spiked in pooled BALB/c sera) were diluted at a minimum dilution of 1/100 in assay buffer and incubated overnight with mIgG1-biotin and mIgG1-digoxigenin (1.25 μg/mL each), during which a portion of ADA present in the sample bound to both conjugated forms of mIgG1 at the same time.

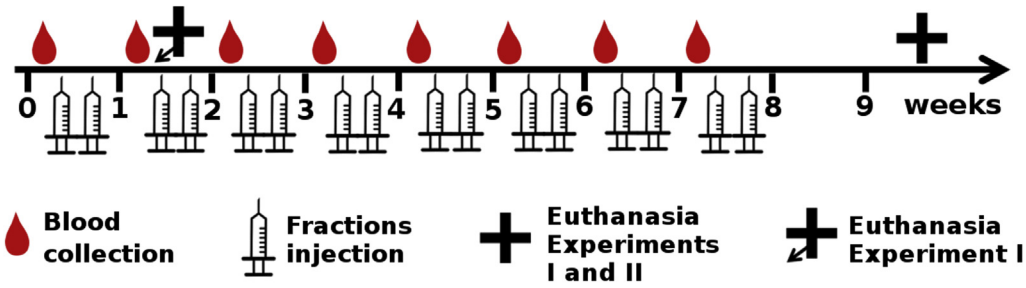

Figure 1. Graphical representation of the administration regimen, blood collection frequency, and experimental end points at which mice were euthanized. ADA detection was performed on blood samples collected at every blood collection time point and during euthanasia. In addition, in experiment II, spleens and draining LNs were collected during euthanasia for the detection of follicular T_{FH} cells.

On the following day, samples were transferred on a streptavidin-coated plate (Thermo Fisher Scientific) and incubated at room temperature for 1 h to capture the ADA-bridged complexes. The plate was washed 8 times with PBST to remove unbound materials. Next peroxidase labeled anti-digoxigenin antibody (Jackson Immunolabs, Suffolk, UK) was added onto a plate to detect bound ADA. The QuantaRed™ Enhanced Chemifluorescent was used as the HRP Substrate Kit (Thermo Fisher Scientific). The fluorescence was exited at 570 nm, and emission at 585 nm was measured with a Tecan Infinity plate reader (Tecan Group Ltd. Männedorf, Switzerland). The signal intensity was proportional to the amount of ADA present in the sample.

The signal of each sample was normalized against background signal for pooled negative BALB/c sera (Bioreclamation, Westbury). Serum was classified as positive if the signal-to-background (S/B) ratio was equal or higher than the cutoff point, defined as the upper 95th percentile of the signal from basal serum samples ($n = 84$ and $n = 64$ for experiment I and II, respectively). All samples found to be positive in the screening assay were tested in a confirmatory assay in which the unlabeled mIgG1 was used as a competitor during the overnight incubation step (see the Supplementary Fig. S2 for further details).

Isotyping

The ADA isotypes in positive sera collected in experiment I at the end of the *in vivo* experiment were measured with a direct ELISA. First, Maxisorp 96-well plates (Thermo Fisher Scientific) were coated overnight with 1 μg/mL mIgG1 in PBS at 4°C. Next, plates were washed 8 times with PBST and blocked for 1 h with 4% nonfat milk (Campina, Zaltbommel, Netherlands) in PBST (PBST/M). After another washing step, sera (100 × diluted in PBST/M) were added into the wells, and the plates were incubated for 1 h at RT with constant orbital shaking. ADA was detected with peroxidase-labeled anti-mouse IgM, IgG2a, IgG2b, and IgG3 Abs diluted 1:2000 in PBST/M (Life Technologies, Rockford). The IgG1 subclass was not measured due to interference of plate coating material (mIgG1) with detection reagents (anti-mouse IgG1 Abs). Next, the plates were washed again and developed with QuantaRed substrate. After incubation for 15 min, the reaction was stopped, and 100 μL of reaction solutions were transferred into the wells of black 96-well plates. The fluorescence was excited at 570 nm and emission at 585 nm was measured with a Tecan Infinity plate reader. The signal of each sample was normalized against background signal for negative BALB/c sera.

Follicular T Helper (T_{FH}) Cell Detection

Single cell solution was prepared by pressing spleens and LNs isolated during experiment II through a Falcon® 70 μm cell strainer (Corning, Amsterdam, The Netherlands). Next, red blood cells present in splenocytes solution were lysed with ACK lysis buffer (Thermo Fisher Scientific) according to manufacturer's protocol. Approximately, 100,000 cells were suspended in cold 100 μL of PBS with 1% BSA (PBSB) and stained with anti-CD3-FITC, CD4-eFluor450, CXCR5-PeCy7 and PD1-PE Abs (all eBioscences, Vienna, Austria) diluted according to manufacturer's protocol. After 40 min incubation, cells were spun down (10 min at 350 × *g*), and unbound detecting reagents were removed by 2 times washing with PBSB. After the last wash step, cells were fixed with 1% polyformaldehyde in PBS and analyzed with FACS Canto II flow cytometer (BD Biosciences, Vianen, The Netherlands). Obtained data were analyzed with FlowJo® X (FlowJo LLC, Ashland). The gating strategy used to detect T_{FH} cells is shown in Supplementary Figure S3.

Statistical Analysis

The potential difference between ADA responses upon injection of different fractions was assessed with 1-way ANOVA with the Tukey's multiple comparison test. Calculations were performed in GraphPad Prism® v.5.02 software.

Results

Detection and Quantification of Aggregates

All fractions tested in *in vivo* experiments were analyzed by using 4 complementary techniques covering a size range from fragments to particles of 100 μm in diameter, that is, dynamic light scattering (DLS), HP-SEC, NTA, and MFI (Fig. 2 and Tables 2 and 3). The collective data were used to quantitatively estimate the mass of protein in the form of fragments, monomers, dimers, oligomers (MW $\leq$ 17·MDa, according to MALLS, see Supplementary Fig. S1), submicron particles (0.1-1 μm) and micron particles (1-100 μm) for the different fractions. The results are summarized in Figure 3. One has to keep in mind that this estimation includes errors due to instrument limitations (e.g., gap or overlap in measured size ranges of used techniques) and assumptions used in the calculations (i.e., spherical aggregates, protein density of 1.4 g/mL, particle porosity of 25%, and lack of interactions with HP-SEC column material). These limitations are probable causes of apparent recoveries that were incomplete (e.g., "SEC oligomers purified") or exceeded 100% (e.g., "Supernatant" and "Pellet") when compared to the actual experimental total mass by microBCA.

The HP-SEC analysis of "Unstressed" and "SEC monomers" (Fig. 2a) confirmed that those fractions were mainly composed of monomeric mIgG1 (97% and 99%, respectively). Both fractions contained, besides monomers, a low percentage of dimers (1.7% vs. 1.5%, respectively, in "Unstressed" and "SEC monomers") and oligomers (0.8% vs. 1.1%, respectively). However, "SEC monomers" had higher estimated amounts of submicron size aggregates than

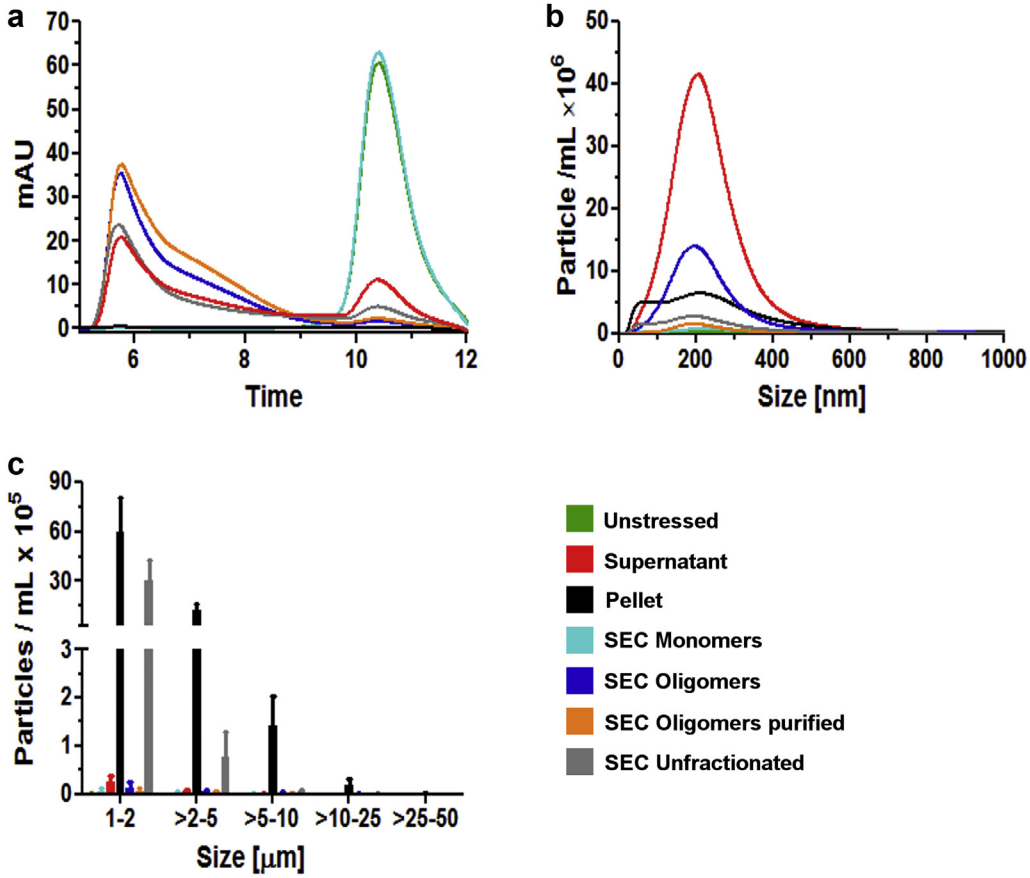

Figure 2. Size distribution of mIgG1 fractions. (a) Representative HP-SEC chromatogram recorded at 280 nm, (b) average submicron particles size distribution of all injected solutions ($n = 16$) measured with NTA and (c) average ± SD micron size particles size distribution of all injected solutions ($n = 16$), measured with MFI.

"Unstressed" (4% vs. 0.5%, respectively, Fig. 2b). Both of those fractions had a very low content of micron size aggregates (0.03% in "Unstressed" sample and 0.3% in "SEC monomers," Fig. 2c). The measured Z_{ave} of "Unstressed" mIgG1 was 12 ± 1 nm (PDI 0.11 ± 0.06), which corresponds with the expected size of monomeric antibody. In contrast, the Z_{ave} of "SEC monomers" was 121 ± 72 nm (PDI 0.25 ± 0.05), most likely due to presence of submicron particles in this fraction.

As expected, the "Supernatant" fraction was found to contain the highest number of submicron size particles among all fractions (Fig. 2b). The Z_{ave} of particles in "Supernatant" was 179 ± 20 nm and the PDI was 0.21 ± 0.01, suggesting a relatively narrow particle size distribution in this fraction. The amount of mIgG1 in submicron particles was estimated to be 135%, which must be an overestimation, also because a substantial amount of oligomers (39%) was observed (Fig. 2a). According to MALLS analysis, aggregates assigned as soluble oligomers consisted of up to circa 100 mIgG1 monomers (data not shown). The estimated content of residual micron size particles found in "Supernatant" was 1.8% (Fig. 2c). Monomeric and dimeric mIgG1 represented 12% and 2.5%, respectively (Fig. 2a).

The "Pellet" fraction was successfully enriched in micron size aggregates corresponding to about 100% of protein (Fig. 2c). The Z_{ave} was determined to be 1867 ± 504 nm (PDI 0.80 ± 0.14). More than 99% of all micron size particles measured with MFI in this fraction had a size below 10 μm. The submicron size aggregates accounted for 57% of total protein (Fig. 2b). This fraction contained hardly any monomers or dimers (both below 0.5% according to HP-SEC, Fig. 2a). Similar to the "Supernatant" fraction, the "Pellet" fraction showed a recovery >100%, indicating an overestimation of

Table 2
Summary of the Results of DLS Measurements of mIgG1 Fractions Obtained for 3 Representative Batches (Average Values ± SDs)

Sample	DLS	
	Z_{ave} [nm]	PDI
"Unstressed"	12 ± 1	0.11 ± 0.06
"SEC monomers"	121 ± 72	0.25 ± 0.05
"SEC oligomers"	97 ± 9	0.22 ± 0.02
"SEC oligomers Purified"	73 ± 4	0.16 ± 0.01
"SEC unfractionated"	103 ± 16	0.30 ± 0.09
"Supernatant"	179 ± 20	0.21 ± 0.01
"Pellet"	1867 ± 504	0.80 ± 0.14

Table 3
Number of Submicron and Micron Size Particles in All mIgG1 Fractions Measured by NTA and MFI, Respectively

Sample	Total Particle Concentration	
	NTA [10^9/mL]	MFI [10^6/mL]
"Unstressed"	0.1 ± 0.7	0.01 ± 0.01
"SEC monomers"	1.67 ± 1.0	0.12 ± 0.10
"SEC oligomers"	25.9 ± 17.3	0.23 ± 0.22
"SEC oligomers purified"	2.4 ± 2.5	0.08 ± 0.09
"SEC unfractionated"	6.7 ± 4.7	34.1 ± 14.5
"Supernatant"	78.6 ± 30.7	0.34 ± 0.15
"Pellet"	21.1 ± 6.3	81.1 ± 27.4

The average number and SD was calculated for all batches of fractions injected into mice ($n = 16$).

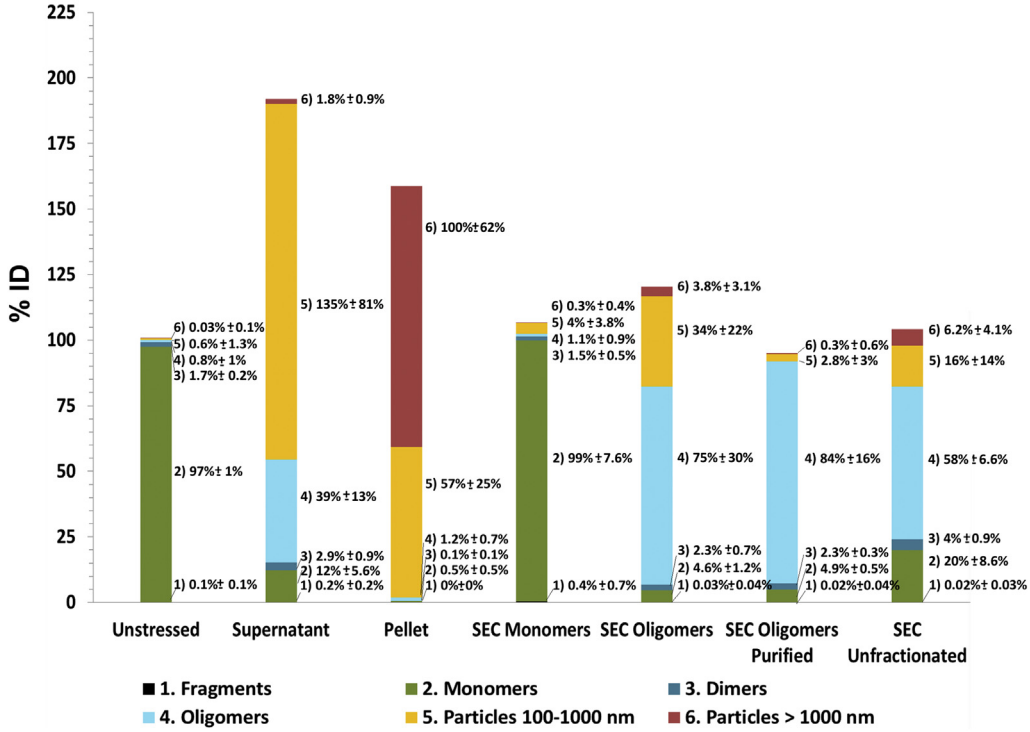

Figure 3. Calculated average mass of mIgG1 species in all batches of injected fractions (n = 16) expressed as % of injected dose (% ID, where 100% equals 10 μg as measured by microBCA).

the amount of protein contained in the submicron and micron particle fractions.

The "SEC Oligomers" and "SEC Oligomers purified" fractions were composed of low amounts of monomers (4.6% and 4.9%, respectively, Fig. 2a). Oligomeric IgG1 was the most abundant protein species in "SEC oligomers" and "SEC Oligomers purified" (75% and 84%, respectively, Fig. 2a). Dimeric mIgG1 represented 2.3% of these fractions (Fig. 2a). In addition, the unpurified "SEC Oligomers" contained submicron and micron size particles (34% and 3.8%, respectively, Figs. 2b and 2c) and the measured Z_{ave} was 97 ± 9 nm (PDI 0.22 ± 0.02). These particulates were successfully removed by filtration and centrifugation, yielding the "SEC Oligomers purified" fraction. The resulting fraction showed a >10-fold reduction in the estimated amount of submicron size particles (2.8%, Fig. 2b) and micron size particles (0.3%, Fig. 2c). Upon purification, the Z_{ave} decreased to 73 ± 4 nm (PDI 0.16 ± 0.01).

The "SEC Unfractionated" sample was estimated to contain 20% of monomers, 4% of dimers, 58% of oligomers (all Fig. 2a), 16% of submicron size aggregates (Fig. 2b), and 6% of micron size aggregates (Fig. 2c). The Z_{ave} was determined to be 103 ± 16 nm (PDI 0.30 ± 0.09). The number of micron size aggregates was comparable to that of "Pellet" (see Table 3); however, they accounted for only about 6% of total protein. This difference in estimated mass can be ascribed to the 10-fold lower number of aggregates larger than 10 μm in "SEC Unfractionated" than in "Pellet."

Structural Characterization of Protein in Unstressed and Stressed Samples

SDS-PAGE

SDS-PAGE analysis under nonreducing conditions showed mainly monomeric mIgG1 for all the fractions (Fig. 4a), indicating that most of the aggregates created during stress treatment were noncovalent. The sample results were impacted by glycosylation as expected in terms of band broadening and migration that was slightly retarded compared to the reference ladder. In all fractions enriched in aggregates, additional bands of covalent aggregates were found (Fig. 4a). These were most likely composed of dimers and trimers. However, as these bands appeared above the marker upper limit, their apparent weight could not be accurately determined. Addition of β-mercaptoethanol to the sample buffer resulted in reduction of disulfide bridges and complete dissociation of monomers and aggregates into light and heavy chains (Fig. 4b).

Western Blotting and Dot Blotting

As depicted in Figure 5, aggregation did not affect recognition of the mIgG1 by anti-mouse total IgG, anti-mouse IgG1, or mIgG1 specific rabbit antibody. This suggests that epitopes present on the monomeric, unstressed mIgG1 were not destroyed by the aggregation protocol. Moreover, it indicates that the aggregated mIgG1 in principle should be capable of inducing antibodies that are cross-reactive with monomeric mIgG1 and therefore should be detectable by our ELISA.

CD Spectroscopy

The influence of aggregation on mIgG1's secondary structure was assessed with far-UV CD spectroscopy (Fig. 6a). The spectra of "Unstressed" and "SEC monomers" showed only minor differences, both being a typical mAb spectrum with β-sheet being predominant in mIgG1's secondary structure.[30,31] Fractions enriched in oligomers and submicron aggregates, that is, "Supernatant," "SEC Oligomers," and "SEC Oligomers Purified" showed significant changes in the spectra when compared with "Unstressed." In all those samples, a circa 2-fold decrease of the negative molar ellipticity at 218 nm was observed. According to Vermeer et al.[31] this indicates loss of β-sheet and increase in random coil structure, and it has been reported for other mAbs upon thermal stress treatment.[30] Because of interference of light scattering by particles, no

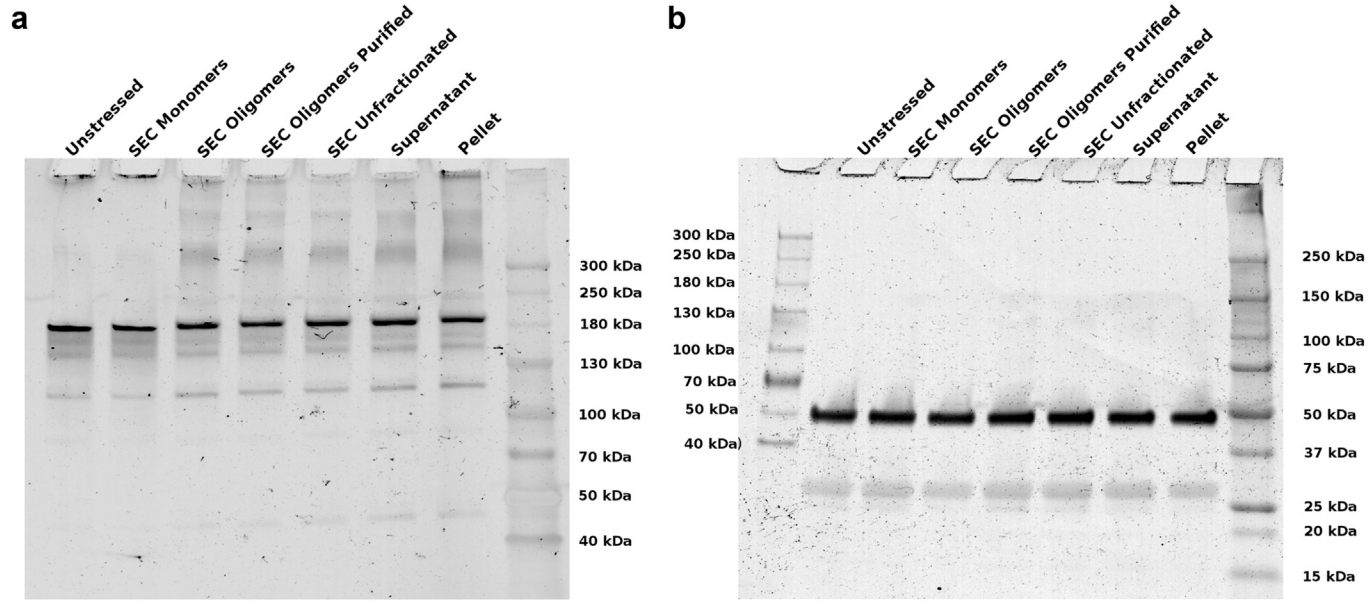

Figure 4. SDS-PAGE analysis of mIgG1 fractions. Samples were analyzed under (a) nonreducing conditions and (b) reducing conditions.

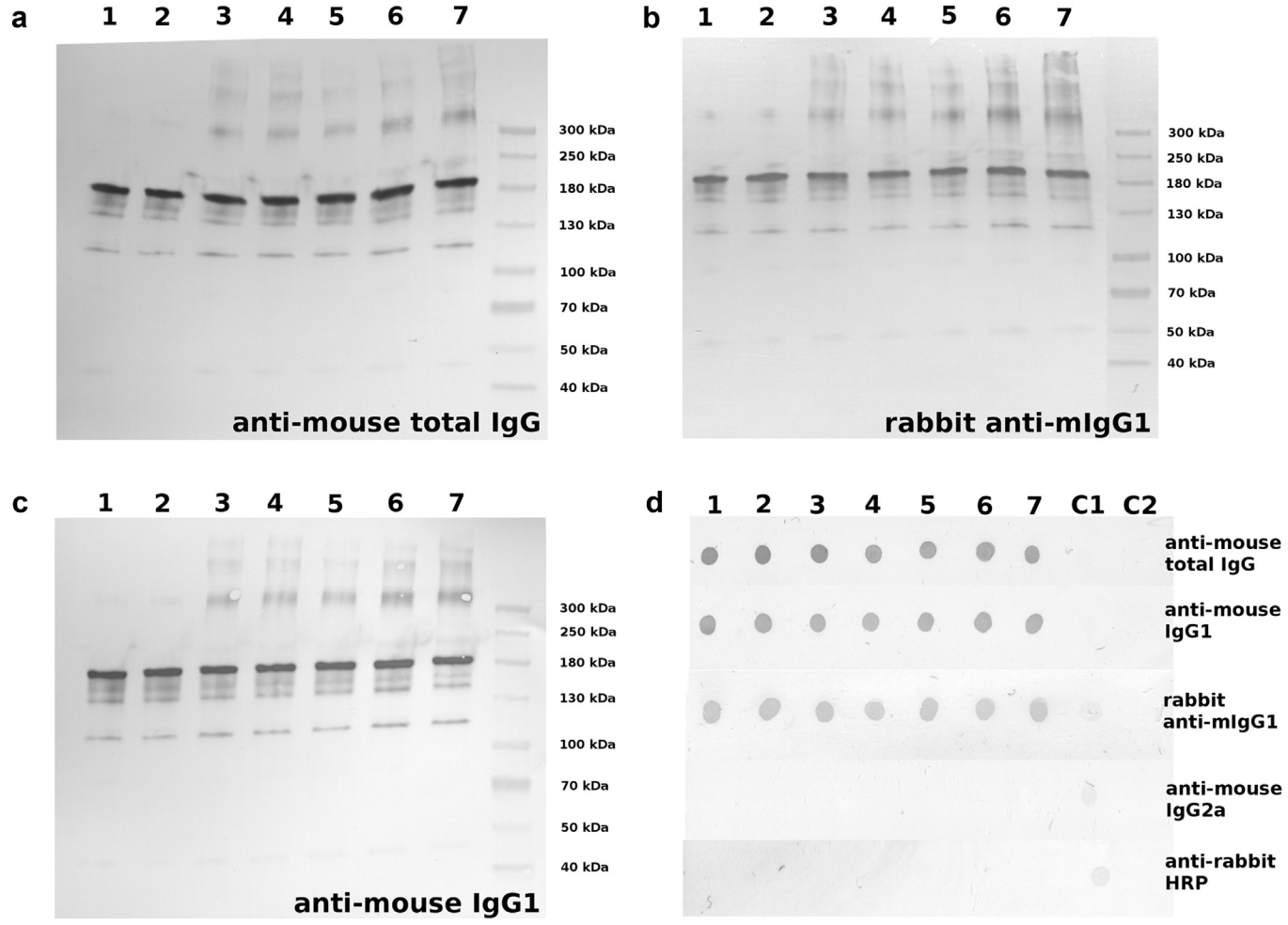

Figure 5. Western blot analysis of the mIgG1 fractions, as detected with anti-mouse total IgG (a), rabbit anti-mIgG1 (b), and anti-mouse IgG1 antibody (c). (d) Dot blot analysis of mIgG1 fractions. The samples are shown in following order: (1) unstressed, (2) SEC monomers, (3) SEC oligomers, (4) SEC oligomers purified, (5) SEC unfractionated, (6) supernatant, (7) pellet, (C1) human mAb, and (C2) bovine serum albumin.

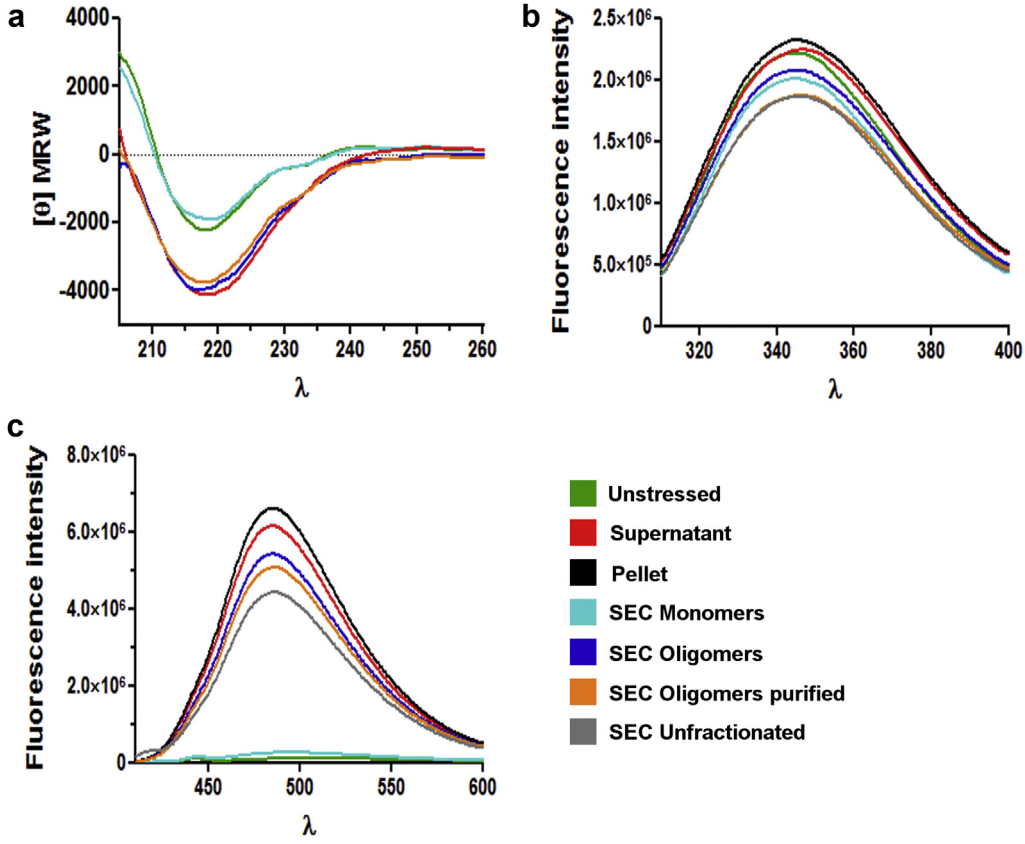

Figure 6. Secondary and tertiary structure analysis of mIgG1 fractions. (a) Far-UV CD spectra, (b) intrinsic tryptophan fluorescence spectra, and (c) fluorescence spectra of Bis-ANS added to the fractions.

meaningful CD spectrum could be recorded for the "SEC Unfractionated" and "Pellet" fractions.

Fluorescence Spectroscopy

The occurrence of potential changes in the tertiary structure of mIgG1 was assessed by measurement of the fluorescence of tryptophan residues. As shown in Figure 6b, no shift in fluorescence emission maximum in "SEC monomers" was detected when compared to "Unstressed" control. Both fractions showed a maximum fluorescence intensity at 344.5 nm. The fractions enriched in aggregates displayed a small red shift in fluorescence emission maximum, ranging from 0.5 nm ("SEC Oligomers") to 2 nm ("Supernatant" and "SEC Oligomers purified"). The intensity of tryptophan fluorescence in fractions obtained with centrifugation, that is, "Supernatant" and "Pellet," was similar to that of "Unstressed" control. However, all fractions isolated via SEC, that is, "SEC monomers," "SEC Oligomers," "SEC Oligomers Purified," as well as "SEC Unfractionated" showed a lower fluorescence than that of "Unstressed" mIgG1. This lower fluorescence intensity and red shift in fluorescence maximum suggest a slightly higher degree of conformational changes for mIgG1 fractions obtained by SEC isolation than for fractions obtained by centrifugation.[32]

In addition to intrinsic fluorescence of mIgG1, fluorescence of Bis-ANS, an extrinsic probe, was used to detect potential changes in tertiary structure. The fluorescence intensity of Bis-ANS significantly increases in a hydrophobic environment. As shown in Figure 6c, the intensity of Bis-ANS fluorescence was similarly low in fractions containing monomeric mIgG1, that is, "Unstressed" and "SEC monomers." In contrast, the Bis-ANS fluorescence intensity increased significantly in the presence of all fractions enriched in aggregates. The fluorescence of Bis-ANS in the presence of "Supernatant" or "Pellet" fractions was higher than in presence of the "SEC Oligomers," "SEC Oligomers Purified," or "SEC Unfractionated" fractions, indicating higher exposure of hydrophobic patches in fractions obtained by centrifugation.

Liquid Chromatography Coupled With Mass Spectrometry

Tryptic peptide mapping by liquid chromatography coupled with mass spectrometry analysis revealed that stress conditions applied in order to induce aggregation did not result in major chemical modifications. "Unstressed" mIgG1 contained some PTMs, that is, low levels of oxidized and deamidated amino acid residues in the heavy chain (Table 4). The levels of these PTMs were similar or only slightly increased for the stressed samples (Table 4). Importantly, no new modifications were detected in any of the aggregate enriched fractions.

Immunogenicity of Aggregates

In experiment I, the immunogenicity in mice of all tested fractions was studied. As shown in Figure 7a, the tested fractions yielded significantly different ADA responses ($p < 0.0001$). The most immunogenic fraction was the "Supernatant," that is, the submicron particle-enriched fraction: 58% of the mice (7 out of 12) treated with this fraction developed measurable levels of ADA after 8 weeks of treatment. The "SEC Oligomers" fraction induced ADA in 16% of the mice (2 out of 12). Only 1 of 12 mice treated with the "Pellet," "SEC oligomers Purified," and "Unfractionated" fractions showed an ADA response. Interestingly, the ADA was first detected after 4 weeks (8 injections) of treatment (see Supplementary

Table 4
Results of Peptide Mapping With Liquid Chromatography Coupled With Mass Spectrometry Analysis of the mIgG1 Fractions

Sample	% Peak Area					
	Oxidation					
	Heavy Chain				Light Chain	
	M34, W36	*W47*	*W110*	*M255*	*W37*	*M49*
"Unstressed"	0.9	ND	ND	1.1	ND	ND
"SEC monomers"	1.1	ND	ND	1	ND	ND
"SEC oligomers"	2.0	ND	ND	2.8	ND	ND
"SEC oligomers Purified"	2.1	ND	ND	3.0	ND	ND
"SEC unfractionated"	1.2	ND	ND	1.2	ND	ND
"Supernatant"	ND	ND	ND	1	ND	ND
"Pellet"	0.8	ND	ND	1	ND	ND

Sample	Deamidation					Isomerization	
	Heavy Chain					Heavy Chain	
	N52N53N54(G)	*N74(S)*	*N84(S)*	*Q112(G)*	*N387(G)N392N393(Y)*	*D62(S)*	*D108(S)*
"Unstressed"	1.3	ND	ND	ND	5.1	ND	ND
"SEC monomers"	1.4	ND	ND	ND	5.5	ND	ND
"SEC oligomers"	1.1	ND	ND	ND	5.7	ND	ND
"SEC oligomers purified"	0.9	ND	ND	ND	4.9	ND	ND
"SEC unfractionated"	1.0	ND	ND	ND	6.0	ND	ND
"Supernatant"	1.1	ND	ND	ND	4.5	ND	ND
"Pellet"	1.3	ND	ND	ND	4.8	ND	ND

Fig. S4). No ADA was detected in mice treated with "Unstressed" control and "SEC monomers." In sera of all positive mice, both IgG2a and IgG2b, but no IgG3 or IgM ADA were found (Fig. 7b).

In experiment II, the immunogenicity of the most immunogenic fraction, "Supernatant," was investigated in a dose-response study. As depicted in Figure 8a, the administered dose influenced the ADA development in between studied groups ($p = 0.015$). The lowest dose induced ADA in the majority of mice, that is, 10 of 12. The treatment with 10 and 30 μg of "Supernatant" triggered ADA in 5 and 2 mice per group, respectively. Moreover, the dose also influenced activation of T_{FH} cells in spleens and LNs on both early-stage and late-stage of immunogenicity development. On day 10, so before the development of ADA could be detected, the number of T_{FH} cells in spleens and LNs of mice administered with "Supernatant" seemed to be decreased when compared with "Unstressed" control. However, the difference was statistically significant only for the dose of 30 μg per injection (Supplementary Fig. S5). On day 65, the number of T_{FH} cells in spleen was strongly influenced by differences in treatment (Fig. 8b, $p = 0.0053$). Injection of 3 and 10 μg of "Supernatant" led to increased numbers of T_{FH} cells in spleens when compared with "Unstressed" control. The number of T_{FH} cells in spleens of mice receiving 30 μg of "Supernatant" was similar to that of the control. This trend was not observed in LNs, in which the number of T_{FH} cells was similar among all tested groups (Supplementary Fig. S5).

Discussion

Protein aggregates are considered one of the potential risk factors for immunogenicity. Several studies have correlated the presence of protein aggregates with increased rates of ADA productions.[11,13,15] However, despite years of studies, it is not fully understood why mAb aggregates increase the risk of immunogenicity and how aggregate features influence ADA formation. In this study, we examined how the size of mAb aggregates influences the immunogenicity upon SC administration in mice. To our best knowledge this study was the first one in which the impact of aggregate size on immunogenicity was comprehensively investigated.

Protein aggregates are heterogeneous in size and can vary from small complexes of a few protein molecules up to visible particles. The impact of protein aggregate size on immunogenicity is poorly understood. Several reports tackling this problem have been recently published.[13-16,33] However, as the available data seems to be conflicting, no universal conclusions can be drawn. In many studies, multiple stress conditions were used to obtain aggregates. In the study described by Filipe et al.,[11] recombinant human IgG1 was stressed with 5 distinct stressors. The obtained formulations were tested in immune tolerant mice for immunogenicity. Metal oxidized recombinant human IgG was found to be the most immunogenic even though this formulation contained the lowest number of aggregates among the tested solutions. However, the high number of variables between the formulations, such as different levels of aggregation, aggregate size distribution, possible chemical modifications, and conformational perturbations, makes it is very difficult to identify the main cause of oxidized mAb's immunogenicity. Another common limitation of experiments described in the literature is immunogenicity evaluation of aggregates of relatively similar size (e.g., dimers vs. small oligomers), representing only a narrow range of aggregate sizes that might be found in protein products.[13] Therefore, on the basis of available data, it is impossible to conclude whether a certain size of aggregates is predominantly immunogenic or whether aggregate characteristics other than size are more important.

To eliminate such confounding factors, aggregates tested in our present study were prepared under almost identical stress conditions. For centrifugation-based separation, the mIgG1 used in this study was exposed to a combination of low pH, high temperature, and stirring. Stirring stress has been included in the aggregation protocol to ensure formation of micron size aggregates. However, the presence of micron size particles in samples intended for SEC separation was undesired. Thus, the mIgG1 intended for SEC isolation of aggregates was exposed to low pH and elevated temperature, but the stirring step used for centrifugation based isolation was omitted. Despite the slight differences in aggregation protocols, the obtained aggregates displayed very similar characteristics apart from size. The SDS-PAGE electrophoresis revealed that the aggregates present in isolated fractions were mostly non-covalent assemblies. Fluorescence spectroscopy and CD analyses

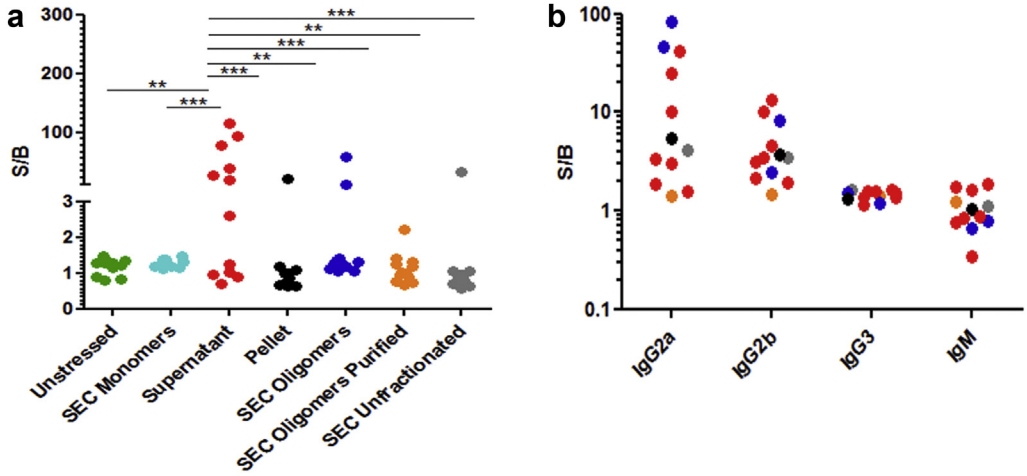

Figure 7. (a) ADA in sera of mice injected with different mIgG1 fractions measured on day 65 (2 weeks post the last mIgG1 injection). (b) ADA isotypes measured in ADA positive sera on day 65. Each dot represents the signal-to-background (S/B) ratio for an individual mouse. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

showed that the structure of mIgG1 in aggregates was perturbed when compared with "Unstressed" control. The analyses indicated loss of β-sheet and exposure of hydrophobic sequences to the solvent, but not complete denaturation. Importantly, no apparent qualitative differences have been found among the different aggregates. Liquid chromatography coupled with mass spectrometry analysis confirmed that mIgG1 did not undergo any major chemical modification during the aggregation procedure. Moreover, Western and dot blotting suggested that all aggregates still contained epitopes found in "Unstressed" mIgG1. Taken together, these results confirm that the major difference between the tested fractions was the size of the mIgG1 aggregates.

The immunogenicity of isolated fractions was assessed in a mouse model. Therefore "Unstressed" mIgG1 was expected to be well tolerated and ADA to be triggered by aggregates. Indeed, "Unstressed" mIgG1 did not trigger ADA in any of mice used in both experiments. Centrifugation allowed separation of 2 fractions: "Pellet," enriched in micron size particles, and "Supernatant," mostly composed of submicron size particles and practically micron particle free. Interestingly, in our mouse model, particles >1 μm in diameter seemed to be poorly immunogenic. This rather unexpected result is in contrast to data reported by Freitag et al.[15] and Telikepalli et al.,[16] who found micron size aggregates to be highly immunogenic. The use of a different protein and different aggregation protocols might explain the differences between studies. Moreover, in the study of Telikepalli et al. the immunogenicity was assessed only in an *in vitro* assay, which might not correlate with production of ADA (the main readout of our study).

In contrast to the "Pellet" fraction, the "Supernatant" fraction induced ADA in most of the treated mice, indicating that in our model, submicron size particles were significantly more immunogenic than micron size ones. The fractions isolated with SEC, that is, "SEC Oligomers" and "SEC Oligomers Purified" were intended to identify the submicron size particles size range responsible for the ADA triggering. When particles bigger than ~100 nm were removed from "SEC Oligomers" by filtration and high speed centrifugation, the immunogenic potential of the obtained formulation ("SEC Oligomers purified") was decreased: only one mouse in this group was found to be ADA positive and the S/B ratio barely exceeded the cutoff point. However, as SEC isolated fractions were poorly

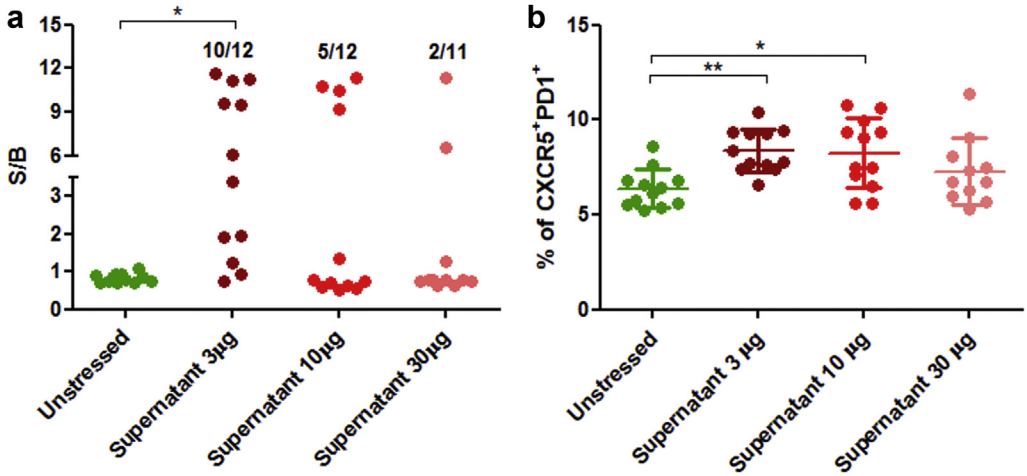

Figure 8. (a) ADA in sera of mice injected with different doses of "Supernatant" fraction. For each group treated with aggregates, the number above the data points indicates the number of positive mice per total number of mice. (b) Number of T_{FH} ($PD1^+CXCR5^+$) cells in spleens expressed as percentage of splenic T helper cell repertoire ($CD3^+CD4^+$). Both measurements were performed on day 65 (2 weeks post last mIgG1 injection). Each dot represents the result for an individual mouse. S/B: signal-to-background ratio. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

immunogenic in our model, future studies are necessary to confirm this observation.

It was suggested that higher immunogenicity of protein aggregates, when compared to monomeric protein, might be a result of their prolonged retention at the SC injection site.[34] Indeed, it has been shown that aggregated proteins persist at the injection site significantly longer than their monomeric counterparts.[23,28,35] In our previous study, we investigated the biodistribution of fluorescently labeled mIgG1 aggregates upon SC injection in mice.[23] We showed that submicron size particles were retained at the SC injection site to a similar extent as micron size ones. Therefore, the difference in immunogenicity of fractions enriched in micron size particles and those containing mainly submicron size particles suggest that retention at the injection site by itself is not sufficient to trigger a strong ADA response. Therefore, the difference in micron size and submicron size particles processing by antigen-presenting cells most likely underlies the observed immunogenicity profile in this case. Studies performed with polymeric particles have shown that nanoparticles are taken up by dendritic cells (DCs) more efficiently than microparticles.[36-38] Although the SC layer has no resident DCs, DCs from surrounding layers might migrate into the SC injection site, where they encounter protein aggregates.[34] More efficient uptake and processing by DCs of submicron size protein particles as compared to small oligomers (<100 nm) or micron size particles would explain the observed high immunogenicity of the fraction enriched in submicron size particulates. However, as protein aggregates are structurally distinct from polymeric particles, future studies are needed to confirm this hypothesis. Moreover, the shape and rigidity of protein aggregates may also affect their uptake efficiency and thereby their immunogenicity, as has been shown for particulate vaccine delivery systems.[39] Another factor possibly contributing to the difference in immunogenicity of submicron and micron size aggregates is potentially different spacing of epitopes on these aggregates. It has been shown that to efficiently crosslink B cell receptors and thereby facilitate antibody responses, epitopes should form a 2-dimensional network with a spacing of about 5-10 nm between the epitopes.[40] As submicron size aggregates might be formed via a different mechanism than micron size aggregates, it is possible that the epitope density of on submicron aggregates of mIgG1 is more favorable for triggering ADA. However, future studies are necessary to confirm this hypothesis.

In experiment II, the influence of the administered dose of "Supernatant," the most immunogenic fraction in experiment I, on its immunogenicity was investigated. Surprisingly, the dose seemed to be negatively correlated with the ADA response and T_{FH} activation in spleens on day 65. This observation might be explained as an effect of tolerogenic regulatory T helper cell epitopes (Tregitopes).[41] We speculate that at low mIgG1 dose, the load of Tregitopes is not sufficient to effectively activate regulatory T cells and thus inhibit ADA formation. However, higher load of Tregitopes during injection of 10 μg and 30 μg of "Supernatant" might overrule the immunogenic potential of increased submicron particles dose, thereby downregulating a T cell−dependent response. A relatively high dose of mIgG1 and consequent immune system "overloading" by particles, might be another explanation for the lower ADA production upon administration of higher doses of "Supernatant." A similar effect has been previously described for IgG-coated glass particles.[42] However, future examination is necessary to fully understand the exact mechanism underlying the low immunogenicity of high doses of submicron size particles.

Although the described results give valuable insight into the impact of aggregate size on immunogenicity, it has to be noted that a direct translation of our findings to humans is not possible. Despite fundamental similarities, the murine immune system differs from the human one and therefore the response observed in mice might not fully reflect the type of response in human patients.[43] For instance, the Balb/c mouse model, although widely used for *in vivo* studies, is inherently biased toward a Th2 type immune response.[44]

Several published reports on immunogenicity of proteins lack extensive characterization of aggregates, especially in the nanometer size range. In some studies the detection of nano size particles was not performed at all.[15] Often these particles have been detected only with DLS or similar techniques.[14,45] However, one has to be aware that when a heterogeneous aggregated protein solution is measured, the accuracy of DLS measurements is low.[46] According to HP-SEC analysis, "SEC monomers" consisted of a similar amount of monomeric IgG1 as "Unstressed" control. However, a small amount of submicron size particles representing at most 4% of protein in this formulation resulted in a significantly higher Z_{ave}, that is, 120 nm versus 12 nm. Moreover, DLS does not provide information on the quantity of particles in the solution. Our data showing high immunogenicity of submicron size aggregates emphasizes the importance of detection and characterization of such aggregates in protein formulations. It is especially important as submicron size aggregates are often formed under a variety of stress conditions.[47-49]

Conclusion

In the presented study we tested the impact of mIgG1 aggregates differing in size on their relative immunogenicity. Importantly, the applied stress conditions allowed generation of differently sized aggregates with otherwise similar characteristics. We found that the fraction enriched in submicron size particles (0.1-1 μm) but not the fractions enriched in micron size aggregates or soluble oligomers, were immunogenic in our model. In conclusion, this study strongly indicates that aggregate size is an important factor influencing immunogenicity of mAbs.

Acknowledgments

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