Pharm Res (2015) 32:430–444
DOI 10.1007/s11095-014-1472-6

RESEARCH PAPER

Investigation of the Immunogenicity of Different Types of Aggregates of a Murine Monoclonal Antibody in Mice

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Received: 30 January 2014 / Accepted: 24 July 2014 / Published online: 15 August 2014

ABSTRACT

Purpose The potential contribution of protein aggregates to the unwanted immunogenicity of protein pharmaceuticals is a major concern. In the present study a murine monoclonal antibody was utilized to study the immunogenicity of different types of aggregates in mice. Samples containing defined types of aggregates were prepared by processes such as stirring, agitation, exposure to ultraviolet (UV) light and exposure to elevated temperatures.

Methods Aggregates were analyzed by size-exclusion chromatography, light obscuration, turbidimetry, infrared (IR) spectroscopy and UV spectroscopy. Samples were separated into fractions based on aggregate size by asymmetrical flow field-flow fractionation or by centrifugation. Samples containing different types and sizes of aggregates were subsequently administered to C57BL/6 J and BALB/c mice, and serum was analyzed for the presence of anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG3 antibodies. In addition, the pharmacokinetic profile of the murine antibody was investigated.

Results In this study, samples containing high numbers of different types of aggregates were administered in order to challenge the *in vivo* system. The magnitude of immune response depends on the nature of the aggregates. The most immunogenic aggregates were of relatively large and insoluble nature, with perturbed, non-native structures.

Conclusion This study shows that not all protein drug aggregates are equally immunogenic.

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KEY WORDS Immunogenicity · Monoclonal antibody · Protein aggregates · Protein particles · Wild-type mice

ABBREVIATIONS

ADAs	Anti-drug antibodies
AF4	Asymmetric flow field flow fractionation
ATR	Attenuated total reflection
AUC	Area under the curve
DMSO	Dimethylsulfoxide
ELISA	Enzyme-linked immunosorbent assay
FNU	Formazine nephelometric units
HRP	Horseradish peroxidase
IR	Infrared spectroscopy
mAb1	Monoclonal antibody
MALLS	Multi angle laser light scattering
PBS	Phosphate buffered saline
RI	Refractive index
UV	Ultraviolet light

INTRODUCTION

Monoclonal antibodies are now the leading category of therapeutic biomolecules and can be used to treat a number of indications, such as cancer, rheumatoid arthritis, Crohn's disease and viral infections [1]. One drawback of this steadily growing class of drugs is their potential to induce unwanted immune responses in the form of anti-drug antibodies (ADAs) directed against the therapeutic antibody. Virtually all protein therapeutics are immunogenic in some fraction of patients [2,3]. The fraction affected varies from a few percent to the majority of patients, depending on the specific protein product and, presumably, its product quality attributes [4,5]. The consequences of unwanted immune responses may be severe, and include alteration of the drug's pharmacokinetics, neutralization of the drug's activity and cross reactivity with and inactivation of endogenous proteins. In turn, these consequences may result in compromised treatment efficacy and patient safety [3,4,6–22].

It has already been reported in literature in the 1960s and 70s that the administration of some aggregated protein products such as plasma protein, human serum albumin (HSA), and γ-globulin to patients can cause unwanted immune responses [23,24]. The immunogenicity of these types of proteins may be related to very high levels of aggregates. While nowadays most protein therapeutics contain very low levels of aggregates, still some products lead to immunogenic reactions in some patients. Therapeutic monoclonal antibodies have shown adverse immunogenic reactions ranging from less than 0.1% to up to 80% of patients [25]. Immunogenicity of products derived from non-human species was thought to be associated with the presence of non-human epitopes, but the immunogenicity of therapeutic monoclonal antibodies has not been reduced with the advent of entirely human therapeutic antibodies [25,26]. Although many factors may contribute to immunogenicity of modern recombinant protein therapeutics, it has been suggested that protein aggregates and other particulate contaminants may play a critical role [27–33].

Many recent *in vivo* studies of therapeutic protein immunogenicity have used transgenic mouse models in which the animals were tolerant to native human protein [31,34,35]. However, the immune response of transgenic animals may be different to that of native animals, as their immune system may be altered due to the transgene. Another approach that circumvents some of the challenges associated with the use of transgenic animals is the use of murine proteins as models of therapeutic proteins for immunogenicity testing in mice. In the current study, we prepared various types and fractions of aggregates of a murine monoclonal antibody and subsequently analyzed and compared immune responses they provoked.

During manufacture, storage and delivery to patients, aggregation of protein therapeutics may result from the formulations being subjected to various stresses including agitation, exposure to light or incubation at elevated temperatures [33,36,37]. We applied these stresses to formulations of a murine antibody in order to obtain five different types of aggregates. The aggregates were analyzed using various analytical techniques, and aggregated samples were fractionated either by centrifugation or by asymmetric flow field flow fractionation (AF4). Subsequently, samples of each aggregate type were administered subcutaneously to C57BL/6 J and BALB/c mice. Mice of the C57BL/6 J strain were selected because the monoclonal antibody investigated in this study was generated in this strain. Additionally, BALB/c mice were chosen because of their well-known strong Th2 immune response, resulting in robust antibody elicitation [38]. Thus, they represent a particularly sensitive animal model. The subcutaneous route of administration was chosen as this is commonly used in patient therapy using monoclonal antibodies [1]. Antibodies directed against the drug as well as the pharmacokinetic profile of the drug were investigated by serum sample analysis.

MATERIALS AND METHODS

Materials

A murine monoclonal anti-mouse TNF-α IgG2c antibody (mAb1) formulated in 20 mM histidine at pH 5.7 and produced in C57BL/6 J mice was kindly provided by AbbVie Bioresearch Center, formerly Abbott Bioresearch Center (Worcester, USA). Histidine, sucrose and hydrochloric acid were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Highly purified water was generated in-house using a PURELAB Plus instrument from USF Elga (Celle, Germany). All reagents were of analytical grade.

Size Exclusion Chromatography (SEC)

Size exclusion chromatographic analyses were conducted on an Agilent 1100 system with a detector for refractive index (RI), as well as multi angle laser light scattering (MALLS) and UV detection (Agilent Technologies, Palo Alto, USA). In addition, a Wyatt Eclipse separation system was connected to the system (Wyatt Technology Europe GmbH, Dernbach, Germany). A Superose 6 10/300 GL column (GE Healthcare, Little Chalfont, UK) was used as the solid phase. Phosphate buffered saline (PBS) at pH 7.4 (12 mM phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride) with a flow rate of 0.5 ml/min was used as the mobile phase. The protein recovery was calculated using the UV absorbance signal at 280 nm. The total area under the curve (AUC) of the unstressed sample was defined as 100%.

Light Obscuration (LO)

Light obscuration measurements were conducted using a PAMAS-SVSS-C Sensor HCB-LD 25/25 (Partikelmess- und Analysensysteme GmbH, Rutesheim, Germany) to quantify particles≥1 μm. Three aliquots of 0.3 ml of each sample were analyzed. Between the sample measurements the system was rinsed with highly purified water until the system was almost free of particles. Before the measurement an inlet flow of 0.3 ml of the sample was flushed and discarded.

Turbidity Measurements

Turbidity was measured by 90° light scattering at λ=860 nm using a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany). Approximately 1.8 ml of the final samples were analyzed according to the European Pharmacopoeia. Results are reported in formazine nephelometric units (FNU) [39].

Endotoxin Testing

The samples were analyzed for the presence of endotoxins using a FDA-licensed Endosafe-PTS instrument (Charles River Laboratories, Wilmington, USA) with a limit of detection of 0.005 EU/ml. The European Pharmacopoeia defines a benchmark in the monograph describing water for injections at 0.250 EU/ ml [40].

Infrared (IR) Spectroscopy

For the determination of changes in secondary structure of the protein, native and aggregated samples were analyzed directly after preparation at 10 mg/ml by IR spectroscopy in attenuated total reflection (ATR) mode. The buffer blank was the formulation buffer without protein (20 mM histidine buffer at pH 5.7). A Bio-ATR II unit of a Tensor 27 spectrometer (Bruker Optics GmbH, Ettlingen, Germany) at 20°C was used. Spectra were collected from 4000 to 850 cm^{-1} with a resolution of 4 cm^{-1}. 120 scans were averaged for each sample measurement. Each spectrum was background corrected and vector normalized on the amide I band. Finally, the second derivatives of the spectra were calculated and smoothed 17 points according to the Savitzky-Golay algorithm.

UV Absorbance Spectroscopy at 280 nm

UV absorbance spectroscopy to determine protein concentration was either performed in a quartz cuvette at 25°C using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Waldbronn, Germany) or in a 96 well-plate made of quartz at 25°C using a FLUOstar Omega instrument (BMG Labtech GmbH, Offenburg, Germany). For each measurement a linear calibration curve including at least three different concentrations of mAb1 was prepared, and the concentration of the samples was calculated based on the Lambert-Beer-law. Each measurement was background corrected.

Preparation of Samples for Injection into Mice

The formulation buffer for the preparation of all samples was a sterile filtered 20 mM histidine buffer, pH 5.7. The initial protein concentration was 10 mg/ml. One milliliter of this solution was filled in 2R vials, glass type 1 (Schott AG, Mainz, Germany) which were closed using Teflon-coated rubber stoppers from West Pharmaceutical Service, Inc. (Lionville, USA).

Eight different sample types were prepared (Table I). The placebo formulation included histidine buffer and 8% (*w/v*) sucrose to adjust tonicity only. The native mAb1 formulation was prepared by diluting the mAb1 stock solution to a final protein concentration of 25 μg/ml using histidine-sucrose buffer. For the preparation of an adjuvant-containing formulation, aluminum hydroxide gel (Alu-Gel-S suspension, Serva Electrophoresis GmbH, Heidelberg, Germany) was added to the protein solution in a 40:1 aluminum hydroxide to protein mass ratio. This adjuvant suspension was gently stirred at 200 rpm for 13 h before injection to permit sufficient time for the protein to adsorb onto the aluminum hydroxide. The final overall concentration in the sample was 25 μg/ml of total protein and 8% sucrose (*w/v*). The adjuvant-containing protein suspension was prepared freshly on the day of administration, as alum suspensions show instability during freezing.

To generate aggregates by light exposure, vials containing 10 mg/ml mAb1 solution in 20 mM histidine buffer pH 5.7 were irradiated at room temperature using a Suntest CPS (Heraeus Holding, Hanau, Germany) equipped with a xenon lamp that generated light in the wavelength range from 200 to 1000 nm. A filter made of sheet glass was installed to obtain a radiation spectrum comparable to indoor light to which a protein may be exposed to during manufacturing. A constant irradiation intensity of 55±5 W/m^2 was determined after the sheet glass filter by an external irradiation sensor. After irradiation for 48 h, large amounts of soluble aggregates were obtained, whereas after 120 h of light exposure large amounts of insoluble aggregates were collected. Soluble oligomers were collected from samples exposed to light for 48 h, and these soluble oligomers were separated by AF4 (see below). The soluble oligomer fractions of 10 injections on the AF4 instrument were collected in glass tubes and concentrated by centrifugation using disposable Protein Concentrators® (Thermo Scientific, Pierce Biotechnologies, Rockford, USA). The concentrator membrane was made of regenerated cellulose having a molecular weight cut-off (MWCO) of 9 kDa and a capacity of 7 ml. Centrifugation was performed at 4000 x *g*, and the concentration of the resulting fractions was determined by UV absorbance spectroscopy. Finally, samples were

Table I Summary of turbidity measurements and particle counts per ml of the different dosing groups investigated in this study at a concentration of 25 μg/ml. Particle counts are expressed as values obtained from three aliquots ± standard deviation

Dosing group	Description	Turbidity [FNU]	Particle counts ≥ 1 μm	Particle counts ≥ 10 μm / ≥ 25 μm
1	Placebo (formulation buffer)	0.60	172 ± 20	21 ± 9 / 5 ± 3
2	Native mAb1	0.80	883 ± 194	5 ± 2 / 2 ± 1
3	Native mAb1 + alum	N/A	N/A	N/A
4	Soluble aggregates from light exposure	2.20	5137 ± 928	27 ± 3 / 1 ± 1
5	Insoluble aggregates from light exposure	0.84	20804 ± 842	59 ± 3 / 2 ± 1
6	Insoluble aggregates from stirring	3.97	107247 ± 787	292 ± 16 / 2 ± 1
7	Insoluble aggregates from agitation	2.25	8827 ± 253	4 ± 1 / 1 ± 1
8	Insoluble aggregates from incubation at 60°C.	1.77	8073 ± 699	14 ± 2 / 1 ± 1

diluted to 25 μg/ml total protein concentration and subsequently stored at −80°C. Insoluble aggregates were also prepared by stirring the protein formulation for 48 h at 25°C and 400 rpm using Teflon-coated stirring bars of 6 × 3 mm in size (VWR International, Darmstadt, Germany). To obtain aggregates produced by agitation, samples were shaken in horizontal circles for 48 h at 25°C and 800 rpm. Finally, the protein solution was stored at 60°C in a drying chamber for 48 h to obtain aggregates by exposure to elevated temperatures.

Each process (stirring, agitation, and incubation at elevated temperatures) was conducted in three different vials which were pooled directly after termination of the stress method. All samples were diluted at a 1:1 ratio with placebo buffer before centrifugation at 4000 x g for 10 min using a Megafuge 1.0 R and the corresponding 7570 F rotor (Heraeus Instruments, Santa Clara, USA). The pellets formed after centrifugation, were re-suspended in the placebo buffer to a final concentration of approximately 25 μg/ml protein. This calculation was based on the concentration of protein determined in the supernatant and the volume of supernatant. All samples except the alum-containing sample were stored at −80°C prior to injection. Prior to injection each formulation was analyzed in triplicate by light obscuration using a Pamas SVSS instrument (Pamas, Rutesheim, Germany) to determine particle load.

Fractionation of Samples by Asymmetrical Flow Field-Flow Fractionation (AF4)

AF4 was used to separate the soluble oligomers generated in the protein formulation by 48 h of light exposure. The separation was conducted on an Eclipse 2 system (Wyatt Technology Europe GmbH, Dernbach, Germany) combined with an auto sampler of the Agilent 1100 series (Agilent Technologies, Palo Alto, USA). The system was additionally connected to detectors for RI, UV and MALLS. A semi-preparative channel (SP1, Wyatt Technology Europe GmbH, Dernbach, Germany) of 245 mm length and 350 μm height equipped with a membrane made of regenerated cellulose with a 10 kDa MWCO was used for separation. The samples were collected in glass tubes by using a Gilson FC 203B Fraction Collector (Gilson Inc., Middleton, USA). The 20 mM histidine buffer at pH 5.7 was utilized as mobile phase. A sample volume of 100 μl was injected into the system and the fractions of ten single runs were collected.

Animals

Female C57BL/6 J and female BALB/c mice (6–8 weeks of age) were obtained from Janvier, Le Genest-Saint-Isle, France. All mice had access to tap water (autoclaved, acidified, pH 2.5–2.8) and food (R/M-H pellets rodent diet, Ssniff, Soest, Germany) *ad libitum*. Mice were allowed at least two weeks to acclimate (predosing period) before the dosing period commenced. Mice were maintained under specific pathogen free (SPF) conditions in groups of 3 to 4 animals in M2-L cages with bedding and environmental enrichment, such as housing and nesting material, to improve physiological and psychological well-being of the animals. The environmental conditions were set at a room temperature of 22°C and humidity of 60% RH. Rooms were set on a 12-h daily light cycle (light from 7 a.m. to 7 p.m.) except as required for sample collection or other study procedures. Ten animals were included in each experimental group.

All animal experiments were carried out in accordance with the international ethical guidelines for the care and use of laboratory animals, and were approved by the local animal ethics committee of the Regierung von Oberbayern (Reference number Az.55.2.1.53-2532.12.11).

Injection Protocol

All formulations were administered subcutaneously to the scruff of the neck at a dose of 5 μg of protein drug per mouse. Each time, 200 μl of the formulations containing 25 μg/ml of protein were injected. Histidine buffer (20 mM, pH 5.7, containing 8% (*w*/*v*) sucrose) served as formulation buffer and

negative control. Injections were carried out on days 1, 2, 4, 6, 13, 20, 27 of the study, followed by a six-week recovery period.

Collection of Blood Samples

Blood samples were obtained from the tail vein by collecting 120 µl of blood on days 1 (prior to any injection), 6, 13, 20, and 27. All mice were sacrificed on day 71 of the study using a lethal dose of anesthetic (Ketamine/Xylazine). Blood was withdrawn from the *vena cava*. Blood samples were collected in polypropylene tubes (no anticoagulant) and immediately centrifuged for 5 min at 4000 x *g* (Eppendorf MiniSpin ®, Eppendorf AG, Hamburg, Germany) at room temperature. The serum was separated and stored at −80°C before analysis.

Determination of Anti-Drug Antibodies (ADAs)

Anti-drug antibodies towards mAb1 were determined in serum samples by an indirect enzyme-linked immunosorbent assay (ELISA) performed in polystyrene MaxiSorp 96-well plates (Nunc, Roskilde, Denmark). In between the incubation steps, the wells were washed with PBS (12 mM phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride), containing 0.05% polysorbate 20 (Sigma Aldrich, Steinheim, Germany). All chemicals were of analytical grade, and all buffers and solutions were prepared using highly purified water (Purelab Plus, USF Elga GmbH, Celle, Germany).

Wells of the plates were coated with mAb1 by incubating 150 µl/well solution of mAb1 (750 ng/ml in PBS) overnight at 4°C. Blocking of remaining binding sites on the surface of the wells was achieved by incubating each well for 2 h at 37°C with 200 µl 1% BSA (Sigma Aldrich, Steinheim, Germany) solution in PBS. The neat serum samples were pre-incubated in 300 mM acetic acid at a pH range of 2.5 to 3.0 for one hour, then the pH was adjusted to 7.4 using 1 M Tris base pH 9.5 (2-Amino-2-hydroxymethyl-propane-1,3-diol, Sigma Aldrich, Steinheim, Germany) and the samples were diluted 1:100. Immediately thereafter, samples containing 1% serum were transferred to 96-well plates and then incubated for 90 min on a horizontal shaker at 500 rpm at RT. ADAs were detected using horseradish peroxidase (HRP) labeled goat anti-mouse antibodies against different mouse isotypes (IgG1, IgG2a, IgG2b and IgG3) at 1:75,000 dilution (Jackson ImmunoResearch, West Grove, PA, USA). Catalytic activity of the bound peroxidase was measured with 0.65 mM hydrogen peroxide (Merck, Darmstadt, Germany) and 0.05 mg/ml 3,3′,5,5′-tetramethylbenzidine (TMB) (Merck, Darmstadt, Germany) dissolved in dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany). The reaction was quenched by addition of 0.5 M sulfuric acid (Mallinckrodt Baker, Deventer, Netherlands). Absorbance at 450 nm was measured using a FLUOstar Omega plate-reader (BMG Labtech GmbH, Offenburg, Germany). Each serum sample for each group for the same time point was tested as n = 3 samples on the same plate. Due to the larger number of samples, several plates had to be used for the different sampling time points.

Plasma samples were defined positive for ADAs when the mean absorbance values were at least three times higher than the 95th percentile value of negative control plasma. For each isotype and mouse strain, serum samples of 24 untreated mice were used for the determination of mean absorbance and 95th percentile of negative controls.

Determination of mAb1 Drug Levels in Serum (Pharmacokinetics, PK Assay)

For the determination of mAb1 levels in serum samples a sandwich-ELISA assay was conducted using MaxiSorp 96-well plates. 150 µl/well of a solution of murine TNF-α (eBioscience Inc., San Diego, USA) at a concentration of 100 ng/ml in PBS were added to each well and used as capture antigen for mAb1 in serum samples. After adsorption of TNF-α, wells were blocked with 1% BSA in PBS and washed. Samples diluted 1:100 in a solution containing 1.08 M sodium chloride, 0.0144 M tri-sodium citrate dehydrate and 0.0011% polysorbate (Sigma Aldrich, Steinheim, Germany) were then added to the wells and allowed to incubate for 2 h at RT. Afterwards, plates were washed and a biotinylated goat anti-mouse-IgG2c antibody (Southern Biotech, Birmingham, USA) was added at a 1:80,000 dilution. A peroxidase-conjugated anti-biotin streptavidin fragment in 1:16,000 dilution (Sigma Aldrich, Steinheim, Germany) was added after further washing steps. The catalytic activity of the bound peroxidase was determined with the substrate H_2O_2/TMB. The reaction of peroxidase and substrate was stopped by the addition of 0.5 M sulfuric acid. The absorbance of samples in the well plates was measured at 450 nm by a BMG FluoStar plate reader. Varying concentrations of mAb1 spiked into C57BL/6 J or BALB/c serum (LPT - Laboratory of Pharmacology and Toxicology, Hamburg, Germany) was used for calibration.

Statistical Analysis

In each group, the formulations were administered to ten mice, and where applicable, the results are expressed as mean ± standard deviation. Statistical analysis was carried by an analysis of variance (ANOVA) and the probability (p) was calculated using Origin 8 (OriginLab Corporation, Northhampton, MA, USA). A probability of > 95% ($p < 0.05$) was defined as significant.

RESULTS

Analysis of Aggregates Prepared by Light Exposure, Stirring, Agitation and Incubation at Elevated Temperatures

The native protein stock solution contained 33 mg/ml of the monoclonal antibody. Using size exclusion chromatography, 98.0±0.4% monomer, 0.9±0.2% fragments and 1.1±0.2% aggregates were observed in the native protein stock solution (data not shown).

After 48 h of light exposure, soluble oligomers were obtained and subsequently directly analyzed and separated by AF4 (Fig. 1). The irradiated sample was composed of 17.4±3.9% monomer, 4.0±0.3% fragments and 74.4±3.9% oligomers with a total soluble protein recovery of 86.9±4.6%, suggesting that some insoluble aggregates were formed which could not be detected by AF4. The fraction eluting between 29.0 min and 35.0 min (illustrated by the dashed lines) was collected. The approximate mean size of the soluble aggregates in this fraction was 20–100 nm, as determined by dynamic light scattering (data not shown). The native mAb1 was also analyzed by AF4 (Fig. 1). A total protein amount of 1 mg of the light-exposed sample and 0.5 mg of the native mAb1 were injected, respectively. Amounts higher than 1 mg or 0.5 mg resulted in overloading effects.

Light obscuration measurements were carried out immediately after aggregate generation or after a storage time of at least 7 days at −80°C.

The highest numbers of subvisible particles were generated by the stirring method, which contained more than 100,000 particles/ml≥1 μm (Table I). The total number of particles/ml of species≥1 μm size in the other samples ranged from ~1000 (native mAb1) to ~21000 (insoluble species from light exposure). The fractionated soluble aggregates generated by light exposure were<1 μm in size, and low numbers of particles in the micrometer range were detected within that sample. None of the formulations contained more than 300 particles/ml larger than 10 μm. The light obscuration measurements were conducted at the final concentration of 25 μg/ml and after several days of storage at −80°C. No considerable differences were observed in the particle size distribution and total particle numbers (data not shown) between the samples measured immediately after preparation and after storage.

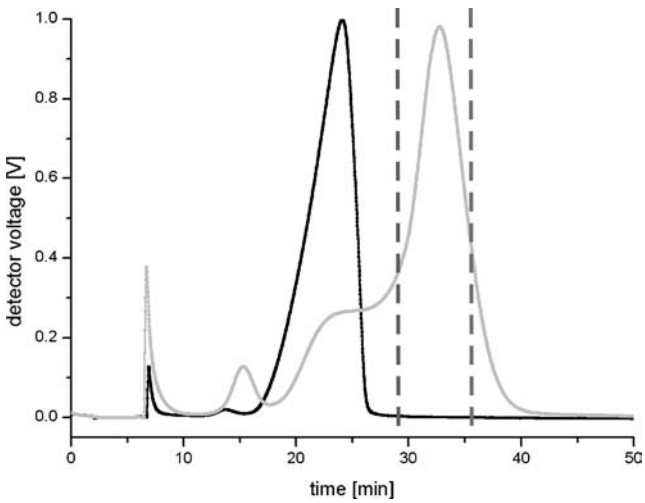

Fig. 1 Fractograms of native mAb1 (*black lines*) and mAb1 after 48 h of light exposure (*grey lines*) recorded at 280 nm. Each sample was injected 3 times. The dashed lines indicate the collected fraction.

Turbidity measurements of the samples at 25 μg/ml protein concentration, before storage at −80°C, were carried out in triplicate and are also summarized in Table I. Slightly increased turbidity values were obtained in the aggregate-containing samples 4, 7 and 8. Stirring (sample 6) resulted in the highest turbidity value of 3.97 FNU, whereas the turbidity of the sample containing insoluble aggregates generated by light exposure (sample 5) is comparable to the solution containing native mAb1.

The formulations were investigated by IR spectroscopy to observe changes in the secondary structure of the antibody after exposure to different stress conditions. The secondary structure of the native and the aggregated mAb1 samples was investigated by IR after the completion of the stress procedure, but not of the final injection samples, since the final concentration of 25 μg/ml of total protein is too low for IR analysis. Figures 2a and b show the 2nd derivative IR spectra of the mAb1 containing formulations directly after preparation. The sample containing protein plus alum was not investigated by IR spectroscopy. The IR spectra shows that processing conditions such as stirring and agitation do not lead to a detectable alteration of the secondary structure of the antibody (Fig. 2a), as the spectra are very similar to those of the native mAb1.

In contrast, light exposure as well as storage at elevated temperatures of 60°C altered the conformation of the mAb1 and resulted in a strong decrease of the intramolecular β-sheet band at around 1638 cm^{-1}. Concomitantly, the absorbance of the intermolecular β-sheet bands around 1622 cm^{-1} showed a strong increase for the light-exposed samples and samples incubated at elevated temperatures.

Analysis of Samples Containing Alum as Adjuvant

The monoclonal antibody was adsorbed to aluminum hydroxide (alum) to prepare the adjuvant containing samples. The supernatant of the adjuvant-containing sample after centrifugation was analyzed and 3.0±0.01 μg/ml of mAb1 were

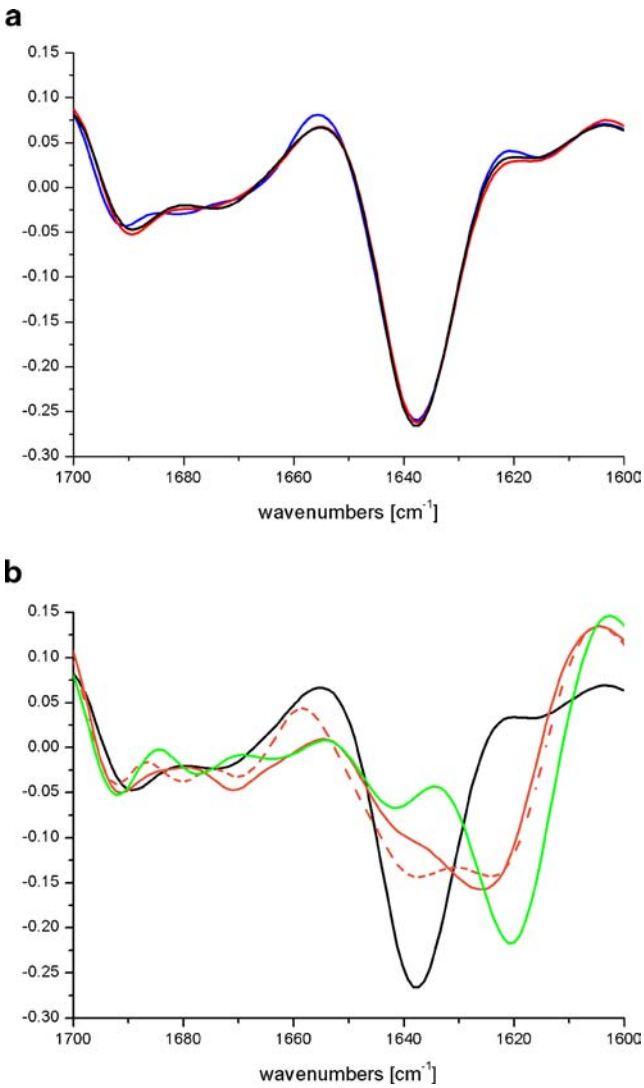

Fig. 2 FT-IR spectra after vector normalization and calculation of the second derivative of the samples. **a**) The black line represents the spectrum of the native mAb1, the blue line represents the spectrum of the stirred sample, and the red line represents the spectrum of the shaken sample. **b**) The light dashed orange line represents the secondary structure of the protein after 48 h light exposure, the solid orange line after 120 h light exposure and the solid green line after storage at 60°C.

detected by UV absorbance spectroscopy. Thus, approximately 22 µg/ml of protein had been adsorbed to the surface of the aluminum hydroxide particles.

Endotoxin Testing

Endotoxin levels below the required benchmark for "water for injection" of 0.25 I.E. as defined in the European Pharmacopoeia were found in all formulations [15]. Endotoxin determination was not possible for the adjuvant-containing sample because its high turbidity interfered with the endotoxin test procedure.

Detection of Immune Response - Anti-Drug Antibodies Directed Towards mAb1

Anti-mAb1 Antibodies in C57BL/6 J Mice

Figure 3 provides an overview of the different classes of IgG antibody isotypes induced in C57BL/6 J mice in response to various formulations of mAb1. In Fig. 3 A results from the IgG1 ELISA are displayed. Due to a high background signal in group 1 (buffer control), the evaluation of the results is difficult. Significant differences between the control groups and aggregate groups were found on day 6, where group 3 (mAb1 + alum) is significantly different from groups 2 (native mAb1), groups 6 (insoluble aggregates from stirring) and group 8 (insoluble aggregates from elevated temperature). ADA results for group 6 also continue to be significantly lower compared to groups 2 and 3 on days 13, 20, and 27, whereas on day 71 differences were not significant.

Figure 3 B summarizes the ADA levels detected for the IgG2b isotype. On day 1 ADA levels from groups 7 and 8 were significantly higher compared to groups 1, 2, and 3 (negative and positive controls). However, on the following sampling days, group 8 was only significantly different to groups 1 and 2 on day 6, but not on the other sampling days. On day 27, group 6 (insoluble aggregates from stirring) is significantly different to the control groups, but not on day 71. On the last sampling day, group 7 displayed the highest ADA value.

Figure 3 C summarizes the ADA levels detected for the IgG3 isotype. On day 13, groups 4 and 7 are significantly different to all control groups, whereas group 6 is only significantly different to group 3. On day 20, all aggregate groups 5, 6, 7, and 8 are significantly different to the buffer control and to the native mAb1, whereas on day 27 only groups 5 and 6 remain significantly different to all control groups.

The data show that the native mAb1 is not immunogenic in the syngeneic C57BL/6 J strain.

Anti-mAb1 Antibodies in BALB/c Mice

Figure 4 provides an overview of the ADAs induced in BALB/c mice. In contrast to the results for the C57BL/6 J strain, mAb1 was robustly immunogenic in the allogeneic BALB/c strain. The ADA isotype with the highest titer was IgG1, followed by IgG2a and IgG2b. The IgG3 isotype ADA levels observed in BALB/c mice were low. Among the various groups, the insoluble aggregates generated by light (group 5) showed the highest IgG1 ADA titer, followed by insoluble aggregates generated by stirring, commencing on day 13 after four injections (Fig. 4a). On days 20, 27, and 71, group 5 showed the highest IgG1 level which was significantly different to all control groups. Also on day 27, groups 6, 7, and 8 were significantly different to the

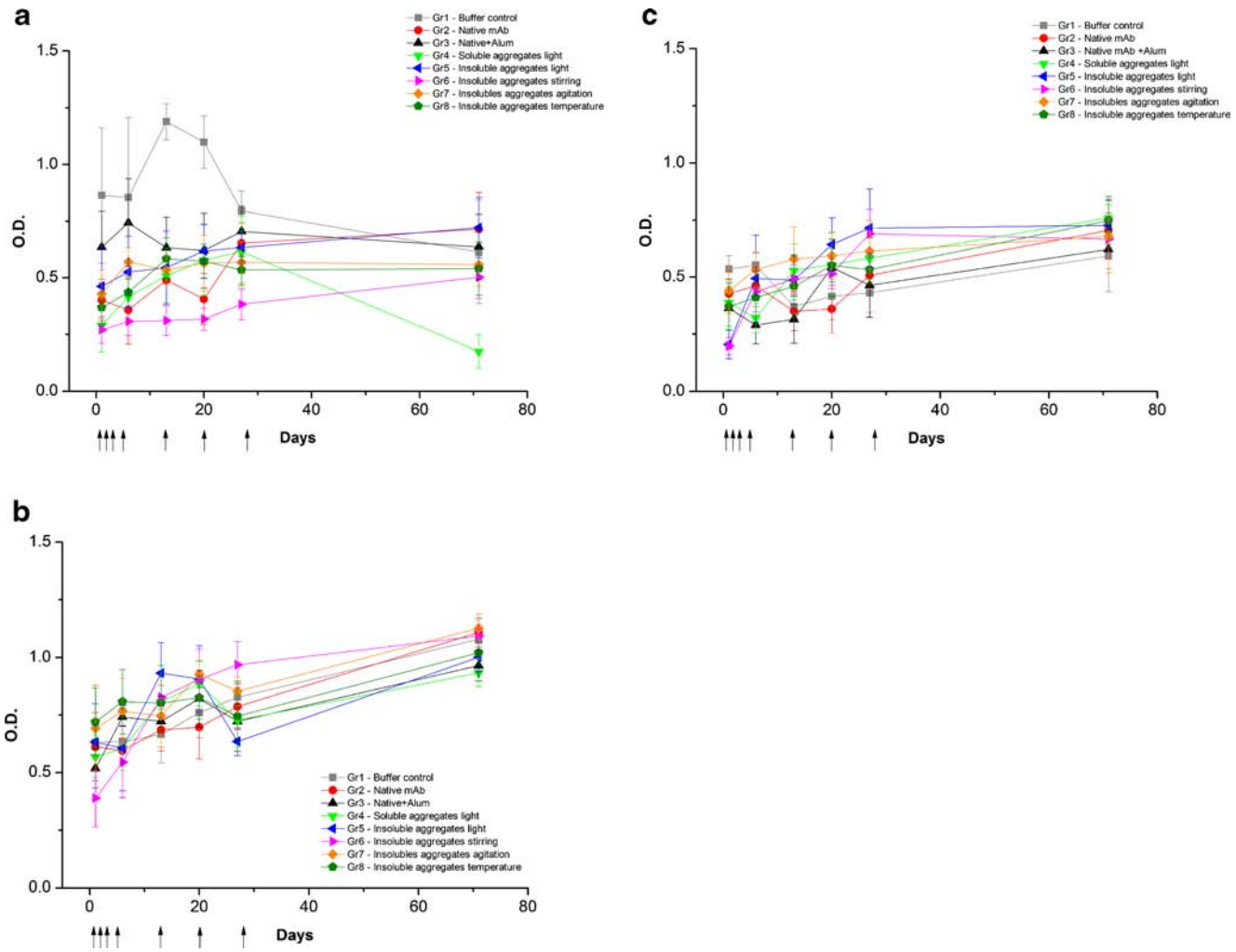

Fig. 3 Overview of the different IgG anti-mAb1 isotype responses in C57BL/6 J mice at the different blood sampling time points, plotted as absorbance units. The arrows represent the injection time points. (**a**) IgG1, (**b**) IgG2b, (**c**) IgG3. The error bars represent the standard error of the mean of ten animals per group.

buffer control and native mAb1. For the IgG2a class ADAs, soluble and insoluble aggregates as well as alum group except light-generated insoluble aggregates induced higher titers than the native mAb1.

Comparison of Responses in C57BL/6 J and BALB/c Mice

As expected the immune response to mAb1 is quite different in the two mouse strains. The allogenic BALB/c did show a significant immune response especially for the IgG1 and to a lesser extend for the IgG2a and IgG2b ADA titer, whereas mAb1 was not immunogenic in C57BL/6 J mice. In addition, aggregates from light exposure, stirring and elevated temperature, that induced weak IgG3 antibodies in C57BL/6 J, completely failed to induce any IgG3 response in BALB/c mice.

Detection of mAb1 Circulating in Blood Stream - Pharmacokinetics

The determination of the pharmacokinetics of a monoclonal antibody is of great importance since it is crucial to determine how fast the drug is cleared from the blood stream in the presence and absence of ADAs. It can be assumed that significant amounts of ADAs can strongly reduce circulation time and serum concentration of the monoclonal antibody, as the likelihood that the drug is captured by the circulating ADAs is increased. An ELISA based on murine TNF-α, the antigen of the mAb1, was performed. The recovery of mAb1 in the formulations prior to injection was determined. In all formulation, except for the formulation containing only insoluble aggregates after light exposure, substantial amounts of mAb1 were quantified using the ELISA method (data not shown).

No quantifiable amounts of mAb1 were found in the serum of animals that had received the placebo formulation or the

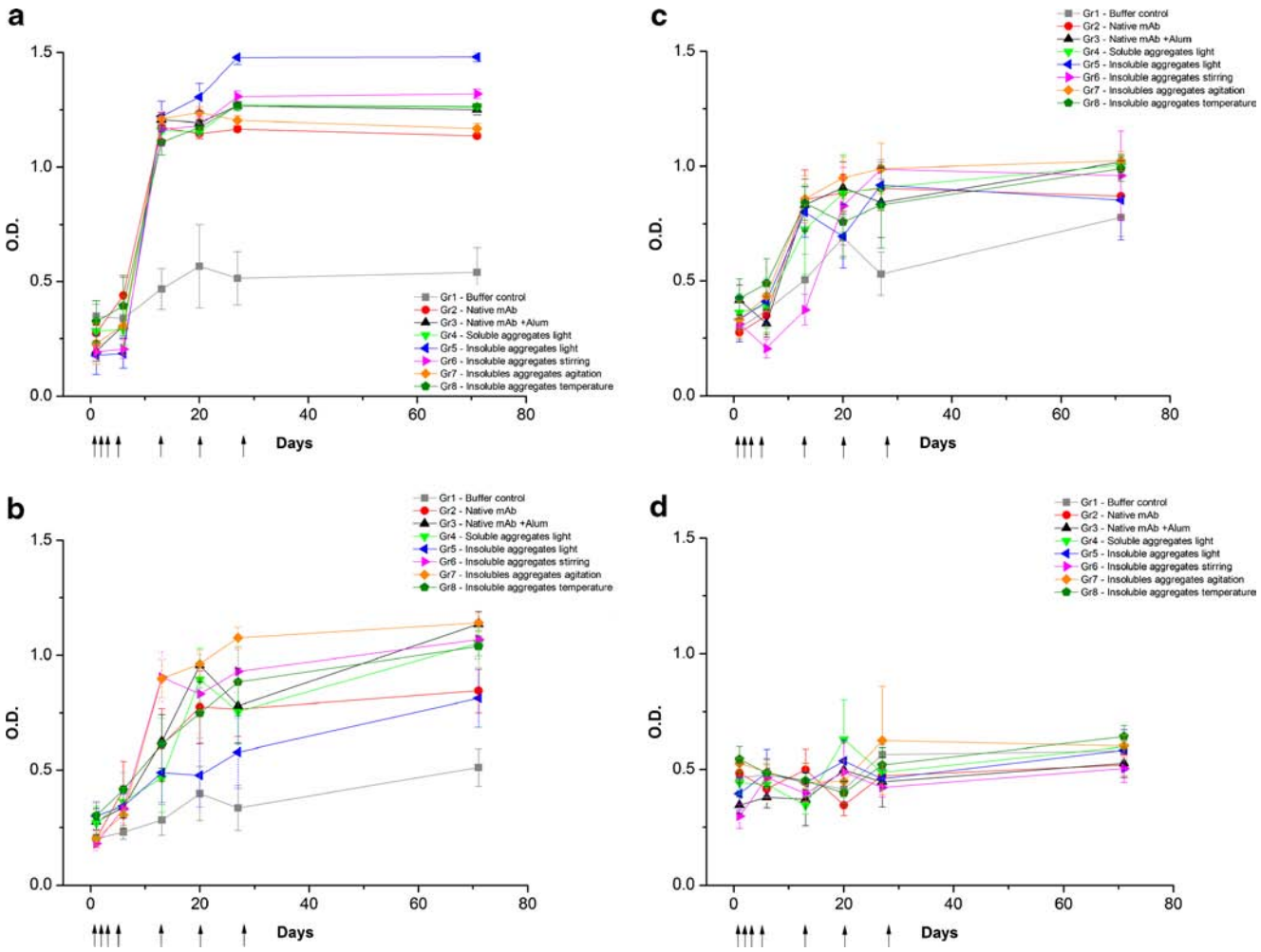

Fig. 4 Overview of the different IgG anti-mAb1 isotype responses in BALB/c mice at the different blood sampling time points, plotted as absorbance units. The arrows represent the injection time points. (**a**) IgG1, (**b**) IgG2a, (**c**) IgG2b, (**d**) IgG3. The error bars represent the standard error of the mean of ten animals per group.

formulations containing the insoluble aggregates. This was observed in both C57BL/6 J and BALB/c mice. Interestingly, in C57BL/6 J mice both native mAb1 formulation and the native mAb1+alum formulation resulted in quantifiable and similar serum levels indicating that mAb1 adsorbed to alum particles was completely released from the particles and behaved like the native mAb1 and addition of the alum adjuvant did not render mAb1 immunogenic in C57BL/6 J mice (see Fig. 5a). The maximum serum levels of mAb1 were measured on day 6, after three booster injections had been administered on days 1, 2 and 4. Later on in the study, injections were given weekly, and subsequently the serum levels decreased slowly and reached baseline levels after the 6 weeks of wash out phase. Since IgG class monoclonal antibodies are known to have half-lives of several days to weeks, the results obtained for these groups are reflecting a regular, unhindered pharmacokinetic profile.

However, the results for the native mAb1 formulation and the adjuvant formulation were considerably different in BALB/c mice, where mAb1 was only detected on day 6. This recovery of mAb1 only on day 6 of the study in BALB/c is similar in mice receiving the native mAb1 or mAb1+alum (see Fig. 5b). Neither after administration of native mAb1 formulation nor after administration of native mAb1 plus adjuvant, circulating mAb1 molecules were detectable at any other bleeding time point than day 6.

The repetitive administration of the formulation containing soluble aggregates (group 4) resulted in no detectable free circulating mAb1 molecules in C57BL/6 J (but there still could be mAb1 complexed to the anti-drug antibodies), whereas levels of mAb1 were detectable in BALB/c mice on days 13 and 20, and these differences were significant to all control groups.

DISCUSSION

The aggregates administered in this study differed in size and structural properties as analyzed by light obscuration,

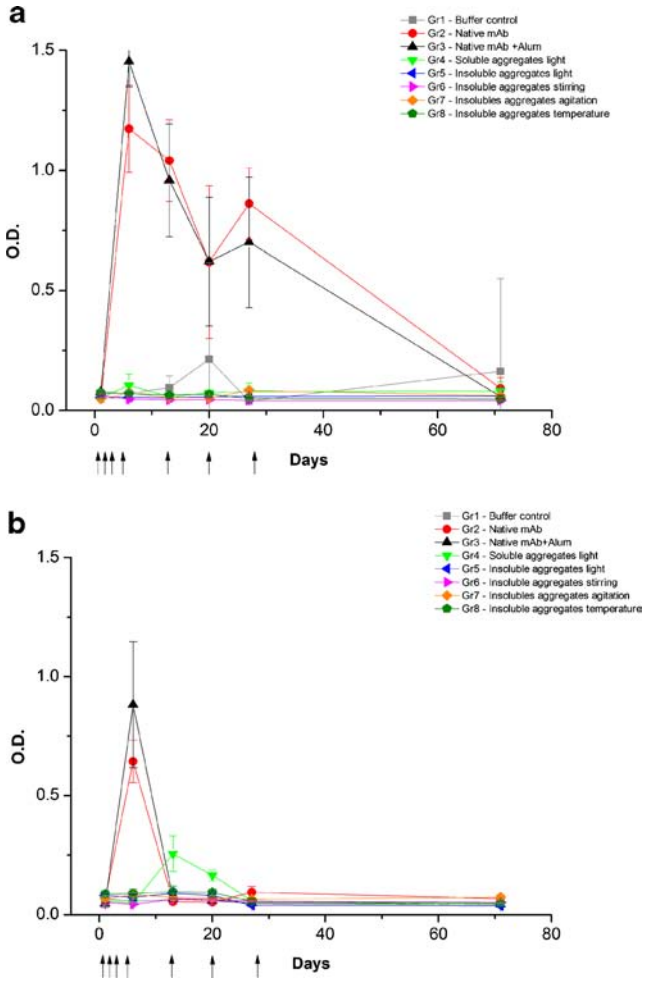

Fig. 5 Absorbance from the PK ELISA in (**a**) C57BL/6 J and (**b**) BALB/c mice. The arrows represent the injection time points. The error bars represent the standard error of the mean of ten animals per group.

turbidity measurements, size exclusion chromatography and FT-IR spectroscopy. According to the immunon model hypothesized by Dintzis *et al.*, aggregates of large sizes and repeating epitopes on their surface are capable of inducing an immune response [41], but the mechanisms behind immune responses to protein aggregates have not yet been clarified. Currently, regulatory organizations and industrial research groups focus on anti-drug antibodies that potentially can have an impact on the efficacy of the drug, altering pharmacokinetics and thus put the success of the therapy at risk. Thus, it is supposed that aggregates with structural properties close to the native protein are foremost crucial in the formation of ADAs. However, it is also entirely conceivable that antibodies towards aggregate-specific structures can be generated as well, and probably do not have a large impact on the pharmacokinetics of the therapeutic protein. They might on the other hand lead to adverse effects such as allergic reactions.

The IR spectroscopy data showed that, of the five formulations containing aggregates, three contained protein species with a strongly modified secondary structure. Exposure to light (resulting in either soluble or insoluble aggregates) as well as storage at elevated temperatures leads to strong changes in the protein structure as indicated by unfolding. In contrast to that, the protein mostly maintained its native secondary structure after agitation and stirring.

In this study, samples were centrifuged and the supernatant was analyzed by IR. Therefore, one needs to consider that the detected non-native like structure could at least partially also reflect changes in the monomer and not only the aggregate.

Unfolding and structural modifications might imply any ADAs induced by these formulations will not recognize the native mAb1 and will not be detected in the ADA assay that utilizes the native mAb1. However all aggregate-test formulations groups scored high and similar signals in the ADA assay indicating that induced ADAs were capable of binding to native antibody molecules.

The type of stresses (stirring, agitation, light exposure, incubation at elevated temperature) chosen for the preparation of the different type of aggregates resulted in reliable and reproducible quantities of these types of different aggregate species. While storage at an elevated temperature of 60°C may not be a relevant temperature with respect to the ICH guidelines, the melting temperature (T_m) of mAb1 was not exceeded.

With regards to the *in vivo* studies, we investigated a murine monoclonal antibody (mAb1) in C57BL/6 J and BALB/c mice. No cross-reactive antibodies against the native molecule were expected in the C57BL/6 J animals, since mAb1 was generated in this mouse strain. A minor response to the administered mAb1 was expected in BALB/c mice [42]. Nonetheless, the generation of anti-drug antibodies directed against aggregates was assumed to be stronger than the native mAb1 in BALB/c mice. Different IgG isotype anti drug antibodies (ADA) were analyzed and compared between the eight different dosing groups, including five aggregate-test groups and three control groups. The placebo and the native mAb1 group served as negative control groups, whereas the mAb1 plus adjuvant-containing formulation served as a positive control for the generation of ADAs, as alum has been described in literature to induce a reliable antibody (Th2) response [43].

Comparison of C57BL/6 J Mice and BALB/c Mice

The repetitive administration of the mAb1 formulations revealed completely different ADA responses in C57BL/6 J and BALB/c mice.

The anti-drug antibody responses towards the control groups substantially differ between the two mouse strains. A high background signal was detected for the placebo formulation in the C57BL/6 J strain, which made analysis of the data difficult. However, with regards to the native mAb1+

adjuvant formulation significant differences were observed between the two strains: BALB/c mice show strongly elevated immune responses (especially of IgG1 isotype) compared to the C57BL/6 J mice that showed no strong response (Figs. 3a and 4a).

The enhanced titers were also observed in the IgG1 isotype ADAs in the BALB/c mice that received aggregate containing formulations. In BALB/c mice, test groups induced higher IgG1-ADA than of the native mAb1 group: insoluble aggregates from light exposure, stirring, agitation, and elevated temperature. For none of these test groups, an IgG1 immune response was detected in C57BL/6 J mice.

Immunogenicity of mAb1 in the Control Groups

As expected, the administration of the formulation buffer (placebo) did not induce significant levels of ADAs in BALB/c mice, whereas some background was visible in particular for IgG1 in C57BL/6 J samples. Since C57BL/6 J has been reported as a mouse strain typically showing cellular immune responses, with only minor elicitation of antibodies, low anti-drug antibody responses to the aggregate containing formulations were anticipated. Confirming the results from pharmacokinetic ELISA, the ADA levels detected in animals that received native mAb1 or mAb1 + alum remained close to the baseline values. To our surprise, the positive control consisting of the native mAb1 plus alum did not result in the expected strong levels of IgG1, IgG2b or IgG3 directed towards mAb1. For future studies, it might therefore be useful to investigate a different adjuvant in combination with the mAb1 in the C57BL/6 J mouse strain.

For BALB/c mice, we expected these animals to recognize the native mAb1 as a foreign molecule. Nonetheless, this mouse strain was included in our study because of its ability to generate strong antibody responses. The exclusive administration of the native mAb1 served as a reference in these animals, since the aim was to detect increased immunogenicity of formulations containing aggregates. This assumption was supported by the increased levels of IgG1, IgG2a and IgG2b after administration of the formulation containing the native mAb1. They were all significantly higher than those after administration of placebo formulation. The positive control including alum resulted in increased levels of IgG1-ADA and to a lesser extent of IgG2a and IgG2b-ADAs in comparison to native mAb1. Anti-mAb1 IgG2a levels were determined only in the BALB/c mice, since C57BL/6 J mice lack the IgG2a immunoglobulin gene [42].

Soluble Aggregates of mAb1

The soluble oligomers formed after light exposure and separated by field-flow fractionation were supposed to be smaller in nature than the insoluble species. The stability of those soluble aggregates during storage had been shown in previous studies [44]. They are also known to be of reversible constitution and tend to dissociate back into monomeric species when stored in an unfrozen state. It can be assumed that such weakly associated (dissociable) aggregates will not exist for a long time after subcutaneous injection. However, the exposure to light may have induced some chemical alterations and oxidation processes to the molecules that might still be present in the structures after dissociation. The administration of soluble aggregates after light exposure resulted in an IgG1-ADA profile with increasing absorbance ratios and a drop during the six-week recovery phase in C57BL/6 J mice. However, as the buffer control resulted in the highest absorbance values these differences are not significant (Fig. 3a). The ADA profiles of IgG2b and IgG3 induced by the same aggregate species did not show these tendencies. The increase in ADAs within the first four weeks of the study were slower than for IgG1 and no drop was detectable after the recovery phase, suggesting that a potential elimination due to mAb1 complexation is sustained as well.

Insoluble Aggregates of mAb1

Insoluble aggregates are very large particles composed of associated protein molecules. They can be either visible or subvisible and some tend to precipitate, while others do not. The insoluble aggregates investigated in this study were all separated from soluble species by centrifugation. The absorption or degradation of subcutaneously administered aggregates, especially the particulate structures, has not yet been reported in literature. The differences in the ADA isotype profiles might arise from the sustained absorption to the lymphatic system and are probably not related to a lower immunogenicity. The absorbance obtained in our study cannot easily be converted into total concentrations of anti-drug antibodies, since no appropriate reference was available. This lack of appropriate references is a disadvantage of using a murine mAb1 in a wild-type mice model instead of using a human mAb1 in transgenic mice. However, one can distinguish between the magnitudes of the absorbance to rank the immunogenic potential of those aggregates. The most crucial aggregates in this study therefore appear to be insoluble aggregates. Especially those generated by light exposure are able to rapidly induce ADAs.

A.) Aggregation by light exposure

The insoluble aggregates after light exposure showed substantial changes in their secondary structure. The results of the ELISA used to determine the recovery of mAb1 in the injected samples indirectly confirmed these changes, since the mAb1 in this formulation was the only one of the stressed protein species that was not at all able to bind TNF-α in the PK assay. It can be suggested that

the complementarity determining region (CDR) was structurally modified due to light irradiation and therefore the elicited ADAs are not directed towards CDR. Results from mass spectrometry furthermore indicate that those aggregates possess chemically altered sequences and oxidized patches in the CDR region of the antibody (data not shown). Hence, other insoluble aggregates with a more native-like structure were expected to be more immunogenic in the sense that they could induce ADAs directed towards the native mAb1.

Surprisingly, the insoluble aggregates generated by light exposure rapidly induced the formation of ADAs, even significantly exceeding the titers of ADAs after administration of the positive control on days 20, 27 and 71 (see Fig. 4a). These results indicate that aggregates from light exposure with chemical and structural changes induce binding antibodies towards mAb1, and thus can also have an impact on pharmacokinetics and efficacy. Compared to the soluble aggregates generated by light exposure, all generated ADAs persisted over the entire study. This suggests a critical impact of the size of the aggregates or the more pronounced structural changes on the induction of ADA.

B.) Aggregation by mechanical stress

Two other formulations that induced ADAs of many IgG isotypes contained aggregates from stirring or agitation. In both formulations, the native structure of mAb1 was maintained, but subvisible particle formation occurred. The formulation obtained by stirring, contained the highest numbers of subvisible particles compared to all other formulations. The aggregates generated by agitation resulted in lower numbers of subvisible particles detectable by light obscuration and induced significant amounts of anti-mAb1 antibodies in BALB/c mice, and to a lesser extent also in C57BL/6 J mice, mainly with regards to IgG2b and IgG3. This indicates a relation between the presence of subvisible particles in a formulation and the ensuing immune response. Since the induced IgG1 and IgG2a ADAs for these aggregates generated by two different methods were of similar magnitude in the BALB/c mice, no ranking with respect to immunogenicity aspects could be made for stirring or agitation induced aggregates.

C.) Aggregation by incubation at elevated temperature

The aggregates generated by storage at 60°C possess the most distinct altered secondary structure of all mAb1 formulations. At the same time, this formulation shows fewer subvisible particles≥1 μm compared to all other insoluble aggregate formulations. The aggregates produced at high temperatures showed only a minor immune response compared to the response to the native mAb1 in BALB/c. In C57BL/6 J mice, slightly enhanced IgG3 levels were detected after administration of these aggregates on day 20, with a further increase over time. The strong structural changes within the aggregates might have impeded the formation of antibodies directed towards the native structure of mAb1 and thus only minute ADAs were detected. Within the ELISA format to determine the recovery of mAb1 in the formulations a marginal binding to TNF-α was detected. This was not surprising due to the heavy structural changes discovered in those aggregate species.

For the duration of the study, the animals were maintained in SPF conditions. Despite the repetitive administration of the native mAb1 to the host strain, meticulous cleaning procedures and preparation of all instruments and samples achieved a low level of background. The results obtained in C57BL/6 J mice indicated that native mAb1 was not immunogenic in this mouse strain, regardless of the administered type of formulation. This finding is in agreement with data we observed in another study [45]. However, these mice are also known to elicit fewer antibodies when compared to other mouse strains [46]. This study determined the generation of antibodies directed against the native mAb1, but not towards the aggregates administered to the mice. Therefore, the results do not reflect the total levels of IgG ADA in the serum samples, but the levels specifically directed towards the mAb1 molecule.

It has already been reported in the literature that immune responses of C57BL/6 J and BALB/c mice differ between the strains [46]. The most obvious reason is the known preferential Th2 response in BALB/c mice. Hence, there may be more than one explanation for the varying IgG profiles detected in the two strains. The foreignness of mAb1 to BALB/c is probably one reason for the higher absorbance ratios in the ADA assays and the completely modified pharmacokinetic profile. Strain-specific parameters such as distinctions in the response mechanisms of the immune system are suggested to have a major impact.

Furthermore, the differences discovered in the isotypes of generated ADAs within one mouse strain are well-known phenomena. Interestingly, the IgG1 isotype is the one of highest isotype elicited in the BALB/c mice (the preferential Th2 responders), whereas C57BL/6 J (the preferential Th1 responders) primarily elicit ADAs of IgG3 isotype. Coutelier *et al.* reported in 1988 on the dependence of IgG subclass generation on the type of antigen in mice. Antibodies towards proteins and carbohydrates were found to be restricted to the IgG1 and IgG3 subclass, whereas antiviral antibodies predominantly belonged to the IgG2a subclass [47]. A predominant mechanism of Th1 immune response implies the release of IFN-γ and enhances the secretion of IgG2a [48]. However, since C57BL/6 J are not able to generate IgG2a subclass [42], these isotype immunoglobulins could not be investigated in this study. Instead, IgG3 preponderated in this mouse strain. In BALB/c mice, being predominantly Th2 responders, the

cytokine IL-4 is mainly secreted leading to strong IgG1 responses [49]. Although the Th2 mechanism is preferred, a minor Th1 response will be present as well, inducing the IgG2a subclass. In BALB/c mice, the dominating IgG subclass was also shown to be dependent on the route of administration [50]. However, throughout this study, the samples were administered subcutaneously and therefore no impact on the IgG subclass was expected.

None of the investigated aggregate formulations was capable to induce all of the investigated isotypes, though conversely none failed to induce formation of ADAs. The differences in the aggregate features therefore seem to result in different profiles of immunogenicity and in diverging immunoglobulin patterns. Hermeling *et al.* also observed remarkable differences in the immune response of various aggregates of recombinant human interferon alpha 2b in wild-type mice. In their study, metal catalyzed oxidation, incubation at elevated temperatures and pH variations were used to generate samples with different aggregation levels, and all aggregates increased the immune response in the wild-type mice. The study did not investigate the subvisible particle content in detail, but the DLS results also point towards particulate structures in the immunogenic aggregate samples obtained by oxidation and pH shift [51]. In another study of our group, the impact of microparticulate contaminants in a formulation of a murine monoclonal antibody on the immunogenicity in wild-type mice was highlighted. A considerable enhancement of immune response to the mAb has been observed, indicating that the level of subvisible particles should be minimized in therapeutic formulations [45].

Additionally, the immune responses to the aggregates having a substantially altered secondary structure of the protein vary considerably. The aggregates from stirring and agitation, which retained a substantially native-like structure, resulted in similar responses in BALB/c mice, but not in C57BL/6 J mice. Therefore, more detailed information on the aggregates is preferably needed to explain the differences in their immunogenic potential.

In order to investigate potential differences between the different aggregate species, a large number of aggregates was chosen to challenge the *in vivo* test system and make differences between the aggregates visible. Therefore, the level of aggregates in each of the tested formulations was considerably higher than one would observe in marketed products.

ELISA Format

An indirect ELISA format including an acid dissociation step to dissociate the ADA-mAb1 complexes was used as described before [45]. This type of ELISA allowed detecting different IgG subtypes. Even though we used an established ELISA protocol, the background signal was not as low as expected. The reason for the increase in background signal over time is unclear and remains to be investigated.

Pharmacokinetics of mAb1

As expected, no mAb1 was detectable in the serum of the mice after administration of the placebo formulation (buffer only). Differences were observed between the pharmacokinetics profiles of the native drug and native drug plus adjuvant in C57BL/6 J mice *vs* BALB/c mice (Fig. 5). Administration of the native mAb1 or native mAb1 + adjuvant showed the expected unhindered PK profile by reaching a maximum serum level on day 6 which subsequently decreased till day 71. High recovery levels of mAb1 in the sera of C57BL/6 J mice receiving the native mAb1 formulation or the native mAb1 + alum formulation indicated no substantial ADA formation. If ADAs were present, the immune complex formation would have interfered with the detection of mAb1.

In comparison, in BALB/c mice the maximum serum levels of mAb1 were also observed on day 6; however by day 13, drug molecules were no longer circulating (Fig. 5b). This PK profile strongly suggests the formation of anti-mAb1 antibodies after administration of native mAb1 or mAb1 + adjuvant. The induced ADA molecules probably captured the circulating mAb1 molecules and the formed immune complexes were rapidly eliminated. It is know from literature that the generation of antibodies by the human immune system requires several days [52], which has also been reported for the murine immune system [53]. Thus it is likely that the formation of ADAs is the reason for the inability to detect mAb1 after day 6 in BALB/c mice.

Interestingly, within each mouse strain the PK profiles are similar between the animals that received the native mAb1 and the animals that received the native mAb1 + alum. Alum was not capable of eliciting ADA formation in C57BL/6 J mice, presumably due to their preferred Th1 response [38]. In BALB/c mice the native mAb1 as well as mAb1 + alum elicited ADAs. However, the recovery of mAb1 in the serum on day 6 was slightly higher in the adjuvant group compared to the native group. This finding may suggest that the progress of generation of ADAs started earlier in mice receiving the native mAb1. A slower desorption of the protein adsorbed to the aluminum hydroxide particles and thus a delayed recognition by the immune system may be one possibility.

Low but detectable amounts of circulating mAb1 were detected only in serum following the administration of soluble aggregates after light exposure. Interestingly, BALB/c mice that received this formulation showed a slowly decreasing pharmacokinetic profile starting from day 13. For comparison, in C57BL/6 J mice, these soluble aggregates induced ADA formation and thus only minor mAb1 levels were detected on day 6 (Fig. 5b).

A second aspect which could impact the PK profile is protein bioavailability upon subcutaneous injection. As shown in Table I, stressed formulation contained micron-sized aggregates which – due to their size – are unlikely to be taken up by the lymphatic system or the blood stream. An explanation could therefore be that upon injection a depot of protein aggregates was formed and therefore the pK profile may be altered. An altered disposition of protein after s.c. application has been reported by Filipe *et al.* [54] and Kijanka *et al.* [55] using *in vivo* fluorescence imaging. Both authors report that the biodistribution of s.c. injected significantly differed from unstressed protein [54,55].

CONCLUSION

This study showed that immune response to a murine antibody in mice depended on both the aggregate type and on the strain of mice that they were tested in. Though the genetic backgrounds of C57BL/6 J and BALB/c mice differ only marginally, their immune response in terms of the generation of anti-drug antibodies were substantially different, highlighting the potential influence of patient genetics on immune responses to therapeutic proteins. Aggregates were produced by exposing the proteins to the stresses of light, elevated temperatures, stirring, and agitation. Each of these stresses is commonly encountered during manufacture and use of therapeutic proteins. All of the types of aggregates tested provoked the generation of ADAs, but the levels and isotypes varied depending on the type of aggregate that was tested. Presumably, the characteristics (e.g., size, solubility, extent of chemical modification, conformation) of the aggregates influence their pharmacokinetics and immunogenicity after subcutaneous injection. In the future, detailed tracking studies to determine the *in vivo* fate of protein aggregates would be useful in order to understand the immune responses that they elicit. In this study, the most immunogenic aggregates seem to be relatively large and insoluble, with perturbed, non-native structures. Administration of insoluble aggregates created by exposure of mAb1 to light and stirring lead to rapid formation of high ADA levels. However, even the smaller and soluble aggregates generated by light exposure, which were preliminarily shown to be dissociable, were immunogenic as well. Therefore, the size of aggregates is not exclusively responsible for ADA formation.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors would like to thank AbbVie Inc. for providing the protein and financial support.

Disclosure of Potential Conflicts of Interest Zehra Kaymakcalan and Michael Siedler are employees of AbbVie and are Abbvie stockholders.

The University of Colorado and the Ludwig-Maximilians-University Munich received research funds from AbbVie Inc. (former Abbott Laboratories) to conduct the study.

AbbVie (former Abbott Laboratories) provided financial support, provided the murine antibody used in this study, as well as resources to support the in-vivo studies and the bioanalytical characterization.

Furthermore, AbbVie authors were involved in study design, research, analysis, data collection, interpretation of data, reviewing and approving the publication.

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