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Fate of Multimeric Oligomers, Submicron, and Micron Size Aggregates of Monoclonal Antibodies Upon Subcutaneous Injection in Mice

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ABSTRACT

The aim of this study was to examine the fate of differently sized protein aggregates upon subcutaneous injection in mice. A murine and a human monoclonal immunoglobulin G 1 (IgG1) antibody were labeled with a fluorescent dye and subjected to stress conditions to create aggregates. Aggregates fractionated by centrifugation or gel permeation chromatography were administered subcutaneously into SKH1 mice. The biodistribution was measured by *in vivo* fluorescence imaging for up to 1 week post injection. At several time points, mice were sacrificed and selected organs and tissues were collected for *ex vivo* analysis. Part of injected aggregated IgGs persisted much longer at the injection site than unstressed controls. Aggregate fractions containing submicron (0.1-1 μm) or micron (1-100 μm) particles were retained to a similar extent. Highly fluorescent "hot-spots" were detected 24 h post injection in spleens of mice injected with submicron aggregates of murine IgG. Submicron aggregates of human IgG showed higher accumulation in draining lymph nodes 1 h post injection than unstressed controls or micron size aggregates. For both tested proteins, aggregated fractions seemed to be eliminated from circulation more rapidly than monomeric fractions. The biodistribution of monomers isolated from solutions subjected to stress conditions was similar to that of unstressed control.

Introduction

Monoclonal antibodies (mAbs) are the most rapidly growing group of therapeutic proteins with respect to both the number of newly approved products and current market value.[1-3] They are used as therapeutic agents for treatment of many diseases, such as cancer, rheumatoid arthritis, and inflammatory bowel disease.[2,4,5] They are also employed as targeting moieties for drug delivery systems and radioactive or fluorescent probes.[6,7] Moreover, multiple products derived from mAbs, such as fusion proteins, antibody-drug conjugates, and mAb fragments (e.g., Fabs, nanobodies), have been recently developed.[2,3]

Administration of all biopharmaceuticals, including mAbs, is accompanied by the potential risk of anti-drug antibody (ADA) formation in some patients, which might decrease or even completely block the *in vivo* activity.[5,8-12] Among multiple factors correlated with increased risk of potential for ADA production, the presence of protein aggregates has been widely recognized as an important one.[13] However, despite the large number of studies, it remains unclear why and how they trigger an immune response and which aggregate features are of the highest risk. Route of administration is another factor that has been linked to differences in frequency and magnitude of ADA generation. Historically, the majority of mAbs have been administered intravenously (IV).[2,14]

Abbreviations used: ADA, anti-drug antibody; AUC, area under the curve; GPC, gel permeation chromatography; HP-SEC, high performance size-exclusion chromatography; IgG, immunoglobulin G; IV, intravenous; LNs, lymph nodes; mAb, monoclonal antibody; MFI, microflow imaging; mIgG, murine IgG; NTA, nanoparticle tracking analysis; PBS, phosphate buffered saline; rhIgG, recombinant human IgG; RIPA, radioimmunoprecipitation buffer; SC, subcutaneous; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Conflict of interest: Wim Jiskoot is scientific advisor at Coriolis Pharma, Martinsried, Germany.

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However, an increasing number of mAb products intended for subcutaneous (SC) administration are being developed.[2] In contrast to IV administration, formulations for the SC route might be self-administered, increasing patients' convenience and compliance with prescribed dosing, leading to reductions in the overall therapy costs.[15,16] Bioavailability of protein drugs administered SC, however, is rarely complete, ranging from 30% to 100%, for different products, patients, and sites of injection.[1,16-19] Moreover, it is currently widely held that the SC route results in more immune responses against the drug substance than administration via the IV route.[20-22] However, recent reports have shown that SC administration could actually be less immunogenic than IV in some cases.[23,24]

Protein aggregates may appear in a variety of sizes ranging from dimers in the size range of about 0.01 μm to visible particles of 100s of micrometer in diameter. Very limited data are currently available on the influence of the size of aggregates on their absorption and biodistribution upon SC administration, with only 2 reports describing the fate of aggregated human immunoglobulin G (IgG) and mouse serum albumin upon SC administration.[25,26] Aggregates of both tested proteins persisted much longer than monomers, up to several weeks postadministration, at the SC injection site. An important limitation of these prior studies was the use of unfractionated stressed formulations, which did not allow for a determination of which size range of aggregates was retained at the injection site. Therefore, it still remains unknown how the retention of aggregates at the injection site and their biodistribution are related to their size.

The aim of this study was to improve our understanding of the impact of the size of mAb aggregates on their fate upon SC administration in mice. Moreover, we studied the influence of mAb origin, murine versus human, on the biodistribution by labeling human and mouse IgG1 molecules with an infrared fluorescence dye (IRDye800CW) and subjecting the labeled mAbs to stress conditions to induce aggregate formation. These stressed formulations were then purified to obtain fractions enriched in oligomers, submicron size particles, and micron size particles that were subsequently injected SC in mice. The biodistribution of the aggregate fractions, in comparison with that of unstressed and stressed mAb monomers, was measured *in vivo* and further evaluated by *ex vivo* analysis of selected organs and tissues.

Materials and Methods

Preparation and Characterization of Labeled mAbs and mAb Aggregates

Monoclonal Antibodies

Recombinant mAbs were supplied by MedImmune. Recombinant human monoclonal IgG1 (rhIgG1; pI 9.26) was formulated in phosphate buffered saline (PBS, 1.54 mM KH_2PO_4, 2.7 mM Na_2HPO_4 $7H_2O$, 155 mM NaCl, pH 7.2) obtained from Life Technologies (Paisley, UK). Murine monoclonal IgG1 (mIgG1; pI 6.81) was formulated in histidine buffered saline (25 mM histidine, 150 mM NaCl, pH 6.0). Both model IgGs have targets that are not endogenous to mice. Before labeling mIgG1 with the fluorescent dye, the histidine buffer was exchanged to PBS on PD-10 desalting columns (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol.

IRDye800 CW Labeling

Both human and murine mAbs were labeled at room temperature with IRDye800 CW NHS ester (LI-COR Biosciences, Lincoln, NE), resulting in fluorescently labeled mAbs, further denoted as rhIgG-L and mIgG-L, respectively. The labeling was initiated by adding 50 μL of dye (5 mg/mL in dimethyl sulfoxide) to 1 mL of protein solution (10 mg/mL in PBS). The solution was incubated for 3 h. The reaction was quenched by addition of 0.2 mL of 1 M Tris-HCl, pH 8.2. Free, unbound dye was removed on a PD-10 column with PBS as mobile phase. The average degree of labeling was calculated according to the manufacturer's protocol and was in the range of 0.9-1 dye molecule per protein molecule. The content of free dye in the final formulation was assessed by size-exclusion chromatography (SEC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was below 5% for all batches (data not shown). Far-ultraviolet circular dichroism, differential scanning calorimetry, fluorescence spectroscopy, and surface plasmon resonance analyses indicated that the labeling did not affect the structure of the mAbs (see Supplementary Data "Characterization of Labeled IgGs and Their Aggregates").

Generation and Fractionation of Aggregates

The stress conditions (i.e., sample volume, incubation time, and temperature) were optimized separately for both mAbs in pilot experiments at an mAb concentration of 1 mg/mL (for centrifugation-based separation) and 40 mg/mL (for gel permeation chromatography [GPC]-based separation). To obtain a wide size range of aggregates, rhIgG-L was subjected simultaneously to low pH and elevated temperature, followed by stirring stress. Equal volumes (500 μL) of rhIgG-L solution in PBS (2 mg/mL) and "low pH buffer" (0.1 M Na_2HPO_4, 0.05 M citric acid, pH 4.6) were mixed. The resulting solution was incubated at 62°C for 1 h followed by stirring (30 min, 700 rpm) at room temperature. Next, the pH of the solution was adjusted to neutral with 50 μL of 1 M NaOH. The neutralized solution was centrifuged (3000 g, 10 min, room temperature). "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" for approximately 10 times.

The aggregation protocol described in the previous paragraph was slightly modified to create and fractionate the monomers and oligomers subjected to stress conditions. The rhIgG-L mAb was concentrated to a final protein concentration of 40 mg/mL by using an Amicon® Ultra device (Millipore, Carrigtwohill, Ireland). The aggregation was initiated by mixing 500 μL of concentrated rhIgG-L with 500 μL of "low pH buffer." The solution was incubated at 61°C for 30 min. Directly after heating, the pH was brought back to neutral, the resulting solution was filtered through a 0.22 μm filter (Milipore) ,and fractions were isolated via GPC on an Agilent 1200 system (Agilent Technologies, Palo Alto, CA) equipped with an autoinjector and a fraction collector. Elution was monitored by absorbance at 280 nm by using a Wyatt UV detector (Wyatt Technology Europe GmbH, Dernbach, Germany). Fractions were separated on a High Load Superdex 200 column (GE Healthcare, Buckinghamshire, UK). A volume of 0.9 mL of aggregated solution was injected onto the column, PBS was used as mobile phase, and the flow rate was 1 mL/min. Fractions were collected for 1.5 min between 45 and 120 min of separation. The fractions containing the oligomers (fractions 2-5 and 2-4 for rhIgG-L and mIgG-L, respectively, referred to as "GPC oligomers") and monomers (fractions 16-18 and 13-17 for rhIgG-L and mIgG-L, respectively, referred to as "GPC monomers") were pooled before analysis and injection (Supplementary Fig. S1).

Because mIgG-L was slightly more sensitive to temperature treatment than rhIgG-L, the methods described in the previous paragraphs were adjusted by decreasing the incubation temperature to 55°C to obtain mIgG-L aggregates in a similar size range as those of labeled rhIgG-L (so, incubation at 55°C was used to create fractions isolated via centrifugation as well as via GPC). All other parameters were identical as described for rhIgG-L.