Dose Levels in Particulate-Containing Formulations Impact Anti-drug Antibody Responses to Murine Monoclonal Antibody in Mice

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ABSTRACT: Dosage levels and particulate contents of therapeutic protein formulations are potential factors that impact immunogenicity of protein therapeutics. Here, we evaluated the effect of dose levels on the immunogenicity of protein particulates formed by adsorbing a murine monoclonal IgG2c/κ antibody (mAb1) onto silicone oil microdroplets, glass, or aluminum hydroxide (Alhydrogel®) microparticles. Immune responses to these particulate-containing preparations were compared against responses to solutions of mAb1 that had been ultracentrifuged to minimize particle levels. Formulations containing 5 or 500 μg of adsorbed mAb1 were administered subcutaneously to C57BL/6J or BALB/c mice. Antidrug antibodies (ADAs) were detected using an isotype-specific enzyme-linked immunosorbent assay (ELISA) method or a chemiluminescence method. Sera from BALB/c mice showed greater ADA responses to administration of particles at the 5-μg dose level than at the 500-μg dose level. In sera from C57BL/6J mice, ADA levels detected by ELISA were independent of the particle dose levels tested. ADAs were not detected in sera from C57BL/6J mice performing the chemiluminescence technique. In conclusion, mice administered formulations of a murine antibody adsorbed onto silicone oil microdroplets, glass microparticles, or Alhydrogel® showed greater ADA responses that those that received particle-free mAb1 preparations, and responses were greater for formulations containing lower doses of antibody. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:1610–1621, 2015
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INTRODUCTION

Antigen dose has been shown to impact the resulting immune response. It has been well known for decades that tolerance to an antigen may develop in response to low doses (e.g., low-zone tolerance) or relatively high doses (e.g., high-zone tolerance). For example, in early studies, Dresser[1] observed that higher doses of bovine gamma-globulin (BGG) induced tolerance in CBA mice. However, the minimum dose that is required for tolerance appears to vary from one antigen to another. For instance, doses as low as 50–200 μg of aggregate-free BGG induced high-zone tolerance in CBA mice.[1] In another study, ABC mice injected subcutaneously with bovine serum albumin (BSA) developed tolerance when the dose level was either very low or very high.[2] Tolerance was obtained by injection of doses of about 10 μg BSA (low-dose tolerance) or by injection of about 10–100 mg BSA (high-dose tolerance). However, an intermediate dose (1 mg) induced an immune response. More recently, Braun et al.[3] showed that the immunogenicity of aggregated human INF-α in wild-type or transgenic mice increased directly with the dosing level. Conversely, in a clinical research study of infliximab and adalimumab in Crohn's disease patients, lower doses were reported to induce higher antidrug antibody (ADA) responses than higher doses.[4] Hermeling et al.[5] were also observed a clear interferon-alpha aggregate dose–ADA response effect in the low-microgram dose range in transgenic immune tolerant mice. Unwanted immune responses to therapeutic proteins have been reported for decades.[6,7] Protein aggregates have been shown to play a critical role in such adverse immunogenicity. For example, it was shown that aggregated human gamma globulin (HGG) had a greater potential to elicit immune responses in rats, dogs, or mice than its monomeric form.[8–11] In the these studies, ultracentrifugation of protein samples, which removes protein aggregates and particulates, was reported as an effective means for reducing the level of immunogenicity. The potential of aggregates to contribute to immunogenicity was further demonstrated in recent studies by administering heated or agitated INF-α, INF-β, or rhGH into wild-type or transgenic mice.[12–15] In another recent study, it was reported that aggregates caused by agitation and heating of human IgG1

Abbreviations used: mAb, monoclonal antibody; ADA, antidrug antibody; ELISA, enzyme-linked immunosorbent assay; BGG, bovine gamma-globulin; PBS, phosphate-buffered saline.

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and IgG2 increased the innate and late-stage T-cell immune responses in cultured peripheral blood monocytes.[16] Also, a dose-dependent presentation of antigenic peptides of the surface of antigen presenting cells was observed when the cells were treated with increasing doses of subvisible particles composed of a therapeutic protein.[17] Most recently, Ahmadi et al.[18] used *in vitro* experiments with human CD4+ T cells and dendritic cells to show that minute amounts of subvisible aggregates in formulations of the monoclonal antibody trastuzumab resulted in potent CD4+ T cell proliferation and strong cytokine and costimulatory signals from the dendritic cells.

In addition to particles assembled directly from protein molecules, protein-containing particulates can form because of protein adsorption to foreign materials from container closure, delivery devices, or manufacturing processes.[19–21] Such microparticulate contaminants include glass, stainless steel, tungsten, and silicone oil.[19–22] The current study focuses on glass microparticles, which may be shed from glass vials or syringes, and microdroplets of silicone oil, a common lubricant in prefilled syringes.

The immune response to an administered antigen also depends on recipient genetic factors. In the case of murine models, the strain of mouse used in a study may impact the resulting immune response.[12] For example, BALB/c mice were less easily tolerized to ultracentrifuged human or BGG than were C57BL/6J mice.[23–25] Injection of even very small amounts of ultracentrifuged HGG (as low as 50 μg) induced tolerance in C57BL/6J mice, whereas tolerizing doses in BALB/c mice were as high as 10 mg.[25] It was suggested that BALB/c mice may process trace amounts of aggregates remaining in the samples more efficiently than C57BL/6J mice.[25] Interestingly, when trace amounts of aggregates were removed by salt fractionation, mice from both strains became unresponsive to small doses of HGG. Similar results were observed when single doses of ultracentrifuged BGG were administered to BALB/c and DBA/2 mice.[24] In that study, DBA/2 mice became tolerant at doses of 0.2 mg BGG, whereas BALB/c mice required more than 20 mg to induce tolerance.

In the current study, we used a murine monoclonal antibody (mAb1) as a model therapeutic protein and administered it subcutaneously to either C57BL/6J or BALB/c mice. mAb1 is a murine monoclonal antibody of the IgG2c isotype that binds mouse tumor necrosis factor (TNF). mAb1 is syngeneic to C57BL/6J mice, because it was generated in this strain. In contrast, BALB/c mice do not produce immunoglobulins of the IgG2c isotype,[26,27] which makes mAb1 allogeneic to this strain. Therefore, we expected a stronger immune response to mAb1 in BALB/c than in C57BL/6J mice.

In a previous study, we showed that 50 μg doses of mAb1 were more immunogenic in BALB/c and C57BL/6J mice when the antibody was adsorbed onto microparticles of glass or Alhydrogel®.[28] Alhydrogel® is a microparticulate adjuvant that is commonly used in vaccine formulations. It is known to be effective in provoking humoral immunity by inducing an antibody (Th2) response in the form of IgG1.[29–31] Proteins can be adsorbed to Alhydrogel®, producing particulates roughly 3–4.5 μm in size.[29]

Here, we explored the effect of mAb1 dose levels on immune responses in BALB/c and C57BL/6J mice to mAb1 adsorbed to glass microparticles, Alhydrogel®, or silicone oil microdroplets. We hypothesized that, because of tolerization effects, a higher dose (500 μg) of mAb1 would provoke lower immune responses than a lower dose (5 μg). Although Alhydrogel® is not found in commercial formulations of therapeutic antibodies, we included it in some formulations of mAb1 in order to allow comparison of the effects of glass microparticles or silicone oil microdroplets with those of a particle type known to stimulate immune responses in both humans and in mice of these strains.

The immune response was monitored by measuring titers of various anti-mAb1 antibody isotypes produced in mice after subcutaneous injection of various formulations. Anti-mAb1 antibodies were analyzed by isotype, as described previously.[28,32] Isotype analysis may provide more sensitive ADA detection,[32] especially for cases where the drug itself is an antibody. Furthermore, the isotype of an ADA response may be reflective of the balance between Th1 and Th2 type responses. Titers of anti-mAb1 antibodies were measured with two different methods, an enzyme-linked immunosorbent assay (ELISA) and the Meso Scale Discovery® (MSD)-chemiluminescence assay. Solid-phase ELISA-based assays have long been used and are the most common ADA detection assays. However, recently developed liquid-phase assay techniques such as MSD-chemiluminescence technology may (in some cases) offer a higher sensitivity to detect ADA. In addition during the course of the study, the levels of mAb1 in the murine sera resulting from the various injections were measured to determine the pharmacokinetic (PK) profiles.

MATERIALS AND METHODS

Materials

All chemicals used in this work were of reagent grade or higher quality. Sterile water for injection was used, and all materials used for injection were USP grade. mAb1, a mouse monoclonal antibody (IgG2c/κ, 145 kD) against mouse TNF-α, and mouse TNF-α were provided by AbbVie Bioresearch Center (Worcester, Massachusetts). HRP-goat antimouse IgM, IgG1, IgG2b, and IgG2c were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania). HRP-rabbit antimouse IgG3 was purchased from Fitzgerald Industries International Inc. (Acton, Massachusetts). Silicone oil, Dow Corning 360 1000 cSt Medical grade, was purchased from Dow Corning Corporation (Midland, Michigan), Alhydrogel® was purchased from Brenntag Biosector (Frederikssund, Denmark), L-histidine was from RPI (Prospect, Illinois), sucrose was from Sigma–Aldrich (St. Louis, Missouri), citric acid, trisodium salt dehydrate were from ACROS ORGANICS (Fair Lawn, New Jersey), 3,3′,5,5′-Tetramethylbenzidine (TMB) was from Thermo Scientific (Rockford, Illinois), and sulfuric acid was from Mallinckrodt (Hazelwood, Missouri). Other chemical reagents were purchased from Fisher Scientific (Pittsburgh, Pennsylvania), including sodium chloride, polysorbate 20, and phosphate-buffered saline (PBS; 10x solution, DNase-RNase- and protease-free, 1.37 M sodium chloride, 0.027 M potassium chloride, and 0.119 M phosphate buffer).

mAb1 Stock Solution

A stock solution of mAb1 was received at a concentration of 24 mg/mL in a 15-mM histidine buffer, pH 6.0. Size-exclusion chromatography analysis showed that the stock solution contained 97.3% mAb1 monomer, 2.1% low-molecular-weight species, and 0.6% high-molecular-weight species. The stock