The Role of Protein Excipient in Driving Antibody Responses to Erythropoietin

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ABSTRACT: Human serum albumin (HSA) is an excipient present in formulations of several recombinant protein products that are approved for clinical use. We investigated the relative contributions of HSA and HSA particles to the generation of antibody responses against recombinant human erythropoietin (rhEPO) and the excipient HSA itself. Protein samples were characterized before injection for quantities of monomeric proteins, soluble protein aggregates, and nano- and micron-sized particles. rhEPO, containing various concentrations of HSA particles, were injected three times a week for 8 weeks into mice. Hematocrits and the production of anti-rhEPO and anti-HSA antibodies were determined at various time points. Levels of antibodies against rhEPO in mice injected with HSA-containing rhEPO were higher than those in mice treated with HSA-free rhEPO. Mice injected with formulations that contained particles of HSA produced strong anti-HSA antibody responses; whereas these responses were greatly reduced when particle-free formulations were administered. In contrast, anti-rhEPO antibody responses were not affected by the presence of particles. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:4041–4055, 2015

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INTRODUCTION

The development of stable formulations of therapeutic proteins is critical for the safety and efficacy of these drugs. To reduce protein chemical degradation and aggregation, excipients such as buffering agents, amino acids, sugars and carbohydrates, proteins and polymers, salts, surfactants, chelators, and antioxidants are added to protein formulations.[1,2] Many protein products that are formulated at low drug concentrations—such as recombinant human erythropoietin (rhEPO)—contain human serum albumin (HSA)[3–5] as an excipient that reduces detrimental interactions between drug molecules and surfaces. HSA can also serve as an antioxidant, with its free thiol and methionines acting as scavengers for reactive oxygen species in solution.[6] The HSA used as an excipient in several commercial drug products is purified from human plasma via steps in the Cohn process, including a cold ethanol extraction followed by pasteurization at high temperatures to inactivate viruses.[7] Because proteins are often sensitive to high concentrations of organic solvents and to high temperatures, this processing protocol may be a cause for the elevated aggregate and particulate content found in protein therapeutics containing HSA.[8] HSA aggregates in parenterally administered protein formulations may stimulate immune responses.[8] For example, HSA aggregates have been shown to enhance macrophage uptake, with a positive correlation between the degree of HSA aggregation and increased phagocytosis.[9] This effect is similar to the well-studied adjuvant properties of particulate aluminum salt adjuvants and virus-like particles, which are theorized to enhance immunogenicity, in part, by increasing macrophage uptake.[10]

Every protein therapeutic has the potential to elicit an immune response in patients,[11,12] and many factors, including the presence of protein aggregates and particles, may contribute to the development of antidrug antibodies.[9,13–23] Most studies have focused on the impact of these degradation products on immunogenicity in formulations composed of a single protein.[13,17,23,24] Enhanced immunogenicity because of protein aggregates and particles is mechanistically similar to that of particulate adjuvants, such as aluminum salts,[10] which enhance the immune response in part by increasing uptake and activation of antigen presenting cells (APCs). Once taken up by APCs, the antigen is processed and presented to naïve T helper cells, which in turn can become activated and subsequently activate B cells that display specificity for the same antigen.[25] Also, particles whose surfaces expose repetitive epitopes may engage multiple antigen receptors on the surface of B cells, leading to immune responses that include antibody production independent of T cells.[26–28] As such, particles in therapeutic protein formulations may not only increase APC uptake and possible presentation of antigen to T cells but may also lead to the development of T cell-dependent or T cell-independent antibody responses.

Traditionally, the immune system has been considered to operate on its ability to recognize self from nonself in order to maintain tolerance and identify foreign invaders. Accordingly, T cells that recognize self antigens are deleted in the thymus during their development and do not exist in the periphery,[29] thus preventing T cell-dependent antibody responses to self

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proteins. However, it has been proposed that this model of tolerance does not adequately explain why the immune system launches responses to intracellular components or why adjuvants are needed to elicit responses to foreign proteins. Along with the classical explanation of tolerance, Matzinger[30] suggested that breaking of tolerance is moderated by "danger" signals such as pathogen-associated molecular patterns or aggregated proteins. This model suggests that APCs are activated by danger signals released by cells undergoing stress, damage, or necrosis.[31,32]

Although there are many published studies documenting the role of aggregated protein in driving immune responses, there remains a paucity of published data on the role of proteins added as excipients in stimulating immune responses to the protein drug. However, a recent report from Colino et al.[33] showed that BALB/C mice injected with aldehyde/sulfate latex beads coated with murine serum albumin (MSA) produced CD4+ T cell-dependent IgG responses.[29] Further, these T cells enhanced IgG and IgM responses to capsular polysaccharide, PPS14, when the mice were boosted with latex beads coated with MSA and PPS14.[33]

In the current study, we investigated whether the injection of HSA particles in formulations of rhEPO would result in the development of elevated antidrug antibodies in mice. rhEPO is a glycoprotein that is produced commercially in Chinese hamster ovary cells and is used therapeutically to stimulate the maturation of erythroid precursors in the bone marrow.[34] It has been administered for decades as a treatment for anemia because of chronic kidney failure or cancer therapy. A very small percentage of patients develop immune responses to rhEPO.[35–37] These responses may result in production of neutralizing and cross-reacting antibodies, which may inhibit the biological activity of both the recombinant and endogenous forms of the protein.[37] In severe sustained immune responses, the immunogenicity caused by the drug product has resulted in neutralization of endogenous erythropoietin activity and the development of pure red cell aplasia (PRCA). PRCA is a hematological disorder characterized by progressive, severe, normochromic, normocytic anemia of sudden onset, reticulocytopenia, and a virtual absence of erythroid precursor cells in the bone marrow.[38]

Numerous factors have been postulated as possible contributors to rhEPO immunogenicity. Because the incidence of immune response is so low, inadequate information is available from controlled clinical trials to determine causality. In the late 1990s, there was a spike in PRCA cases in Europe among chronic kidney disease patients who were administered subcutaneous doses of the Johnson&Johnson rhEPO product, Eprex.[39] This event occurred after several product changes were made, including switching from intravenous to subcutaneous administration via a prefilled glass syringe, and a formulation change wherein HSA was replaced with Tween 80®. In 2003, Hermeling et al.[36] suggested that the association of Tween 80® micelles with rhEPO were responsible for the elevated number of PRCA cases. However, in 2005, Villalobos et al.[40] published data that challenged the existence of micelle-associated rhEPO. Another theory proposed that leachates from uncoated rubber plunger stoppers used in the prefilled syringes contributed to rhEPO immunogenicity; however, leachates failed to enhance antibody responses to rhEPO in mice.[41–43] The product was reintroduced to the market following a switch to coated syringe plunger stoppers, and cases of PCRA in patients administered EPREX decreased. In another more recent case report, PRCA developed in two patients treated in clinical trials of the biosimilar rhEPO Binocrit®. The investigation concluded that immunogenicity was because of tungsten-induced protein aggregates in prefilled syringes.[44] Additionally, increased incidence of PRCA was noted in Thailand after introduction of unregulated rhEPO formulations that were reported to contain high levels of aggregates.[45] Schellekens and Jiskoot[46] point out that the contribution of aggregation is consistent with epidemiological patterns of PRCA. The authors argue that product mishandling could have led to more aggregation and that patient self-administration could be associated with higher risk of aggregation (thereby explaining higher PRCA incidences in France where many patients self-administer versus Germany where patients are more often injected by medical staff).

We propose that the presence of HSA particles within rhEPO formulations may act as an adjuvant to enhance both anti-HSA and anti-rhEPO antibody development. To investigate this hypothesis, we compared the antibody responses in mice following injection of a rhEPO product containing HSA particles (HEPO) to the response to a rhEPO product formulated without HSA (EPO), which contained lower quantities of particles and aggregates than HEPO. Also, solutions containing either heat-treated, aggregated HSA, or essentially particle-free HSA were combined with EPO to confirm that any differences we saw in antibody response between the HEPO and EPO products were because of the presence or absence of HSA particles. Additionally, controls containing heat-treated, aggregated MSA or soluble, essentially particle-free MSA were tested to ensure that the antibody responses to HSA-containing samples were not simply because of injection of the xenogeneic human protein into mice. To minimize sample inconsistencies on different injection days, heat-treated samples were exposed to heat stress in a single large batch and frozen as aliquots, which were then thawed as needed for aggregate and particle characterization or injections. Thus, heat-treated samples were exposed to both heat and freeze–thaw stress. In order to correlate their contributions to immunogenicity, aggregate levels and subvisible particle counts and size distributions in all of the protein samples were measured. Samples were injected into mice subcutaneously three times per week for 8 weeks. To track the emergence of immune responses and PRCA, serum samples were obtained at various points during the animal study and analyzed for total anti-rhEPO antibodies, total anti-HSA antibodies, and hematocrit. To gather additional information on immune mechanisms, we performed ELISPOT analysis to enumerate antibody-forming cells (AFCs) in spleens and bone marrow.

MATERIALS AND METHODS

Materials

Epogen® (HSA-containing EPO, HEPO; Amgen, Thousand Oaks, California; lot 1037188 expiry: 01/14) and Albuminar 25 (HSA; CSL Behring, King of Prussia, Pennsylvania) were obtained from a wholesale pharmacy. Erythropoietin bulk drug substance (HSA-free EPO, EPO) was supplied by Hospira (Lake Forest, Illinois). MSA was obtained from Sigma–Aldrich (St. Louis, Missouri). Lyophilized HSA (22060759), used to coat ELISA plates, was obtained from Bioworld (Visalia,