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Review

Immunogenicity of Therapeutic Protein Aggregates

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ABSTRACT

Therapeutic proteins have a propensity for aggregation during manufacturing, shipping, and storage. The presence of aggregates in protein drug products can induce adverse immune responses in patients that may affect safety and efficacy, and so it is of concern to both manufacturers and regulatory agencies. In this vein, there is a lack of understanding of the physicochemical determinants of immunological responses and a lack of standardized analytical methods to survey the molecular properties of aggregates associated with immune activation. In this review, we provide an overview of the basic immune mechanisms in the context of interactions with protein aggregates. We then critically examine the literature with emphasis on the underlying immune mechanisms as they relate to aggregate properties. Finally, we highlight the gaps in our current understanding of this issue and offer recommendations for future research.

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Introduction

Protein aggregation has been defined as the self-association of monomers in their native or partially unfolded forms.[1,2] Many diseases are associated with protein aggregation *in vivo*, including Alzheimer's disease, prion disorders, amyotrophic lateral sclerosis, amyloidosis, Huntington's disease, and Parkinson's disease. Aggregation is also a common instability observed with biologics or protein drug products, can affect efficacy and biodistribution, and may increase the potential for adverse immune reactions in patients.

Aggregates can be produced throughout the life cycle of a protein therapeutic: during upstream and downstream processing, during fill-finish operations, shipping, and shelf-storage, and during handling in the clinic.[3] At the physiochemical level, aggregation can occur through one or more pathways: (1) the self-association of folded proteins, in which aggregates are formed by colloidal interactions with minimal structural change, (2) the self-association of non-native proteins in which the first step is the formation of unfolded or partially unfolded intermediates, and/or (3) covalent reactions among native or structurally perturbed monomers.

Adverse immune responses to therapeutic proteins are well documented, and may manifest clinically as reduced drug efficacy, infusion reactions, cytokine release syndrome, anaphylaxis, or even death.[4] The presence of aggregates in a protein drug product has been associated with an increased potential for these adverse events.[5] Several intrinsic and extrinsic factors are thought to be responsible for the immune response to protein aggregates. Intrinsic factors include aggregate size and amount, as well as the presence[6] and frequency[7] of neo-epitopes on the aggregate surface. Native-like aggregates are proposed to be more immunogenic than those comprised of fully denatured protein,[5] although the underlying mechanism is still unknown. Extrinsic factors such as route of administration, the presence of impurities, dosing frequency, immune tolerance to the monomeric protein, the disease state of the patient, the activity of concomitant immunomodulators, and the immunomodulatory activity of the protein in question can affect the host immune response. Few studies have addressed these issues mechanistically, and there is a dearth of mechanistic literature relating protein aggregates to immunological response both *in vitro* and *in vivo*. There is significant interest on the part of both the biopharmaceutical industry and regulatory agencies in improving our understanding of the factors influencing immune response to protein aggregates, as well as the most appropriate analytical

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methods to monitor aggregate content and to mitigate adverse events.

The problem of immunogenicity of therapeutic protein aggregates has been addressed in a number of key review articles. Based on the then-scarce mechanistic studies, Rosenberg[5] summarized hypotheses of the immunologic effects of therapeutic protein aggregates. With the appearance of many newer studies addressing these issues, Filipe et al.[8] wrote an excellent book chapter that put forward molecular hypotheses regarding the formation of new epitopes on aggregates. Later, Wang et al.[9] and Ratanji et al.[10] provided updated surveys of the preclinical studies on the immunogenicity of aggregates.

In this review, we address the issue of aggregate immunogenicity from a mechanistic viewpoint in light of the results of many key publications that have appeared in the last few years as well as the earlier literature. The focus here is on the immunogenicity of aggregates rather than the clinical immunogenicity of monomeric proteins, about which there are numerous publications. The objective is to survey the available evidence for the immunogenicity of protein aggregates and to identify critical gaps in our understanding, with the hope of improving the safety and efficacy of protein drug products, informing risk assessments and regulatory decision making, and stimulating additional research. We begin with a description of aspects of the human immune system likely to be involved in a response to protein aggregates. With this as background, we then review the available evidence for an immune response to aggregated protein drugs, including clinical trial and postmarketing surveillance data in humans, *in vivo* studies in wild-type (WT) and transgenic (TG) animals, *in vitro* studies using primary cells and cell lines, and the limited literature on *in silico* predictions. We then discuss the limitations of the literature on the immunogenicity of protein aggregates and gaps in our knowledge base, together with suggestions for research to address these limitations. A comprehensive discussion of accelerated stress and analytical characterization methods is beyond the scope of this review; the reader is referred to several excellent recent reviews on the different methods of accelerated stress[3,11] and analytical characterization[12-15] for additional information.

Interactions of Protein Aggregates With the Immune System

The immune system functions through crosstalk of the innate and adaptive systems. The innate system is highly conserved and can rapidly recognize and respond to patterns or repetitive motifs in foreign antigens to provide immediate defense. The adaptive system provides antigen-specific responses through T- and B-lymphocytes. The adaptive response is typically slower than the innate response but can give rise to immunological memory, including the persistence of antigen-specific lymphocytes over decades, which are responsible for a continued ability to respond to the antigen upon repeated exposure. Together, both systems defend the human body against pathogens and other foreign agents.

Innate Response and Antigen Presentation

Although the innate system constitutes the first interface of immunity with protein aggregates, there are few reported studies of the interactions of protein aggregates with innate receptors *in vitro*. Amyloid beta (Aβ) plaques, aggregates of 36-43 amino acid peptides associated with Alzheimer disease, were found to induce TNF-α secretion from monocytes by stimulating Toll-like receptors 2 and 4 (TLR2 and TLR4).[16] Similarly, aggregates of 3 different monoclonal antibodies (mAb) prepared by stirring induced significant increases in the secretion of inflammatory cytokines from peripheral blood mononuclear cells (PBMCs) by stimulating TLR2 and TLR4.[17] In this study, the complement system as well as Fc gamma receptors I and III (FcγRI and FcγRIII) were involved in mediating the innate response. In a separate study using surface plasmon resonance, dimers and multimers of 2 IgG1 (immunoglobulin G) mAbs were shown to have higher *in vitro* binding affinity to FcγRs than their monomeric counterparts.[18] The results also showed a correlation between aggregate size and enhanced binding to the low-affinity FcγRIIA and FcγRIIIB as compared to the high-affinity FcγRI. A similar result has been reported for heat-induced aggregates of IgG1 which had an increased interaction with FcγRs in primary monocytes as compared to monomers.[19]

Developing adaptive immunity by producing antibodies against a foreign agent requires antigen recognition by lymphocytes. T-cells recognize linear epitopes presented by antigen presenting cells (APCs), whereas B-cells recognize conformational epitopes. B-cells, macrophages, and dendritic cells (DCs) are the major APCs that internalize, process, and present peptide epitopes on major histocompatibility complex (MHC) molecules to stimulate naïve T-cells. The maturation of T-cells also requires a second signal delivered by costimulatory molecules that are upregulated on the surface of mature DCs. Upon uptake by DCs, proteins are trafficked to the endocytic compartment where they are digested by proteases, and immunogenic peptides are loaded onto the surface of MHC molecules.[20] MHC-II molecules present peptides from both soluble and particulate extracellular proteins. Similarly, soluble extracellular antigens captured by B-cells are guided to the MHC-II compartment.[21]

Rombach-Riegraf et al.[22] studied antigen presentation of protein aggregates *in vitro*. In their study, IgG aggregates induced the upregulation of monocyte-derived DC maturation markers. Using a combined immunoprecipitation and proteomics approach to identify peptides attached to MHC-II molecules, the authors demonstrated that increases in the amount of protein particles were correlated with increases in the number of peptide epitopes presented by MHC-II molecules. In keeping with these results, Ahmadi et al.[23] reported similar IgG aggregate-induced DC maturation, and showed increased uptake of IgG aggregates by monocyte-derived DCs. The results from both studies demonstrate that aggregation increases sampling of the protein by APCs and hence increases the potential of an immunological response.

Depending on the immune receptor engaged, protein antigens might induce inflammation or immune tolerance. Whether receptor interactions similarly dictate the effects of protein aggregates on immune cells remains less clear. In view of the available information from the aforementioned *in vitro* studies using IgG aggregates, the hypothesized interactions of aggregates with innate receptors, uptake by DCs, and antigen presentation are depicted in detail in Figure 1.

Antidrug Antibody Formation

Generally, antibody formation takes place in either a T-cell dependent (TD) or a T-cell independent (TI) manner. The antibodies formed against the protein molecule are called antidrug antibodies (ADAs). For the purposes of this manuscript, the antigen that elicits ADA formation refers to a protein aggregate that carries one or more T- or B-cell epitopes and should not be confused with the specific target domain of the ADAs. Depending on the epitope, ADAs may have a neutralizing effect on the protein, which may in turn affect its potency, or pharmacokinetics/dynamics, or they may bind to regions of the protein which do not affect safety or potency, with little to no clinical effect. The generation of ADAs can be particularly concerning in the case of therapeutics related to an endogenous protein. In this case, they will also be able to bind to

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