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Effects of Protein Aggregates: An Immunologic Perspective

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Amy S. Rosenberg[1]

[1]Food and Drug Administration, Center for Drug Evaluation and Research, Bethesda, MD

Abstract

The capacity of protein aggregates to enhance immune responses to the monomeric form of the protein has been known for over a half-century. Despite the clear connection between protein aggregates and antibody mediated adverse events in treatment with early therapeutic protein products such as intravenous immune globulin (IVIG) and human growth hormone, surprisingly little is known about the nature of the aggregate species responsible for such effects. This review focuses on a framework for understanding how aggregate species potentially interact with the immune system to enhance immune responses, garnered from basic immunologic research. Thus, protein antigens presented in a highly arrayed structure, such as might be found in large nondenatured aggregate species, are highly potent in inducing antibody responses even in the absence of T-cell help. Their potency may relate to the ability of multivalent protein species to extensively cross-link B-cell receptor, which (1) activates B cells via Bt kinases to proliferate, and (2) targets protein to class II major histocompatability complex (MHC)-loading compartments, efficiently eliciting T-cell help for antibody responses. The review further focuses on protein aggregates as they affect an immunogenicity risk assessment, the use of animal models and studies in uncovering effects of protein aggregates, and changes in product manufacture and packaging that may affect generation of protein aggregates.

Keywords: aggregates, container closure system, immunogenicity, neutralizing antibody, tolerance

Introduction

The immune system has evolved to respond to invasive threats posed by microbial organisms. Such organisms have inherent "signatures" consisting of repetitive displays of proteins, polysaccharides, nucleic acids, or lipids on their external surfaces. In addition, pathogenicity of such organisms depends on complex protein structures critical for invasion and propagation such as enzymes (eg, neuraminidase). The immune system has both an innate response armamentarium, consisting of multiple cellular and humoral pattern receptors, including toll-like receptors (TLRs), which bind to conserved molecular patterns and trigger rapid defense responses, as well as an adaptive immune response arm that responds to unique microbial proteins. Such adaptive responses include pathogen-specific antibody, which may be generated independent of T-cell help (ie, a T helper independent [TI] response) or, less efficiently, may require collaboration with T-helper cells (Th) (ie, a T-helper dependent [TD] response).

An important element of the efficient response to microbial antigens is the rapid production of antibody by B cells that do not require T-cell help. The requirements for generation of antibody to such determinants have been examined in detail for both relatively simple polymers of peptides and polysaccharides, as well as for higher order structures such as viral capsids, composed of repetitive arrays of multiple protein components. Investigating the molecular requirements for TI antibody responses to polysaccharides, Dintzis et al[1] hypothesized that the B-cell stimulatory signal is "quantized" in that a minimum number of cell-surface-expressed antigen receptors must be connected together in a spatially contiguous cluster that they defined as an "immunon." They found that the molecular mass of polymers that successfully triggered TI antibody responses exceeded 100 kDa and that the valence of the hapten moieties exceeded 10. Successive work by many in the field indicated that the generation of a signaling complex leading to B-cell activation and antibody production depended on factors in addition to molecular mass and hapten valency, including hapten affinity, polymer rigidity, and binding kinetics.[2] More recently, Bachmann and Zinkernagel[3] extensively examined the effects of antigen organization on antibody responses by challenging transgenic mice, tolerant to a viral coat protein from vesicular stomatitis virus (VSVgp), with VSVgp in progressively more ordered arrays. Despite tolerance to the soluble monomeric protein, presentation of VSVgp in a loose aggregate was able to generate antibodies in a TD fashion, while its presentation in a highly ordered viral capsid generated antibody in the absence of T-cell help,[3] clearly demonstrating the potency of highly ordered structures in eliciting rapid and efficient immune responses.

Corresponding Author: Amy S. Rosenberg, Director of Division of Therapeutic Proteins, Food and Drug Administration, Center for Drug Evaluation and Research, Building 29A, Room 2D-16, 8800 Rockville Pike, Bethesda, MD 20892. Tel: (301) 827-1790; Fax: (301) 480-3256; E-mail: amy.rosenberg@fda.hhs.gov

The central conclusion of this body of work is that high molecular weight (MW) arrays of antigen are highly efficient in eliciting an antibody response independent of T-cell help, whereas their less ordered counterparts are less immunogenic and may require T-cell help to generate an immune response.

The activation of antibody-producing B cells via the extensive cross-linking of B-cell receptor (BCR) by multivalent ligand involves activation of Bruton's tyrosine kinase/Tec kinase.[4] While this signaling apparatus is of itself sufficient to induce proliferation of B cells, antibody production appears contingent on delivery of a second signal, mediated by Th cells, or potentially by other signaling pathways including those mediated via TLR.[2]

Microbial pathogens express many enzymes and other structures necessary for viral invasion and proliferation. The activity of such pathogenic proteins depends critically on the 3-dimensional conformation of the active site. To protect the host, the immune system makes antibodies that are specific for the active site of the protein (ie, conformation-dependent antibody that neutralizes enzymatic activity). In fact, this preference for antibody to conformational rather than linear determinants of proteins appears to be a general property of the immune system. Thus, even when proteins pose no threat, antibodies are preferentially directed to the conformationally configured portions of the protein. For example, in a recent study by Ito et al,[5] it was found that in mice immunized with isolated hen egg lysozyme (HEL), a complex protein with significant tertiary structure, all of the mAbs elicited (15/15) reacted to native protein, with only 2 of the antibodies also recognizing linear epitopes present in the reduced form, implying that conformational determinants were the binding ligands. The group found that this was the pattern for 4 different nonmicrobial proteins that underwent similar scrutiny.

Additional evidence of the immune system's proclivity for conformation-dependent antibody is found in responses to endogenous proteins such as amyloid proteins. Thus, Nath et al[6] and O'Nuallain and Wetzel[7] found that patients with Alzheimer's disease mounted significant antibody responses to Aβ amyloid structures, but that such antibodies did not bind to the monomeric Aβ amyloid precursor protein. Intriguingly, O'Nuallain and Wetzel found that antibodies to Aβ amyloid structures cross-reacted significantly on amyloid structures from other proteins whose primary amino-acid sequences differed dramatically from that of the Aβ amyloid precursor. Thus, antibody responses to these proteins are specific for common higher-order structural elements. Finally, neutralizing antibody responses to therapeutic protein products appear to be dominated by conformation-specific antibody: serum from 12/13 patients with pure red cell aplasia (PRCA) resulting from antibodies to erythropoietin failed to bind to denatured erythropoietin containing only linear determinants[8]; and neutralizing antibody to human thrombopoietin (TPO) was not inhibited by short peptides of TPO that would have interfered with antibody binding to linear, but not conformational, determinants.[9]

So, how do protein aggregates relate to this "immunologic imperative"? First, we need to define what we mean by protein aggregates. For the purposes of this review, written from an immunologic and not a biochemical perspective, protein aggregates are defined very broadly as high MW proteins composed of multimers of natively conformed or denatured monomers. Such species may be soluble or insoluble (particulate), and reversible or irreversible, within the given environment. Moreover, in keeping with an immunologic focus on the consequences of such species, 3 types of protein aggregates are considered: the first, an assembly of native proteins in a polymeric structure; the second, an assembly of denatured protein irreversibly associated (within the given environment) and dependent on hydrophobic interactions; and the third, covalently linked proteins, which could be either in a native or denatured state.

The hypothesis is that the potency of protein aggregates and, particularly, of particulate protein aggregates in eliciting immune responses pertains to their resemblance to the microbial "signatures" to which the immune system has evolved. Thus, immunity to defined proteins can be enhanced by ensuring aggregation of native proteins in a rigid (eg, virus capsid) type of presentation,[10] an advantageous approach for vaccines. In contrast, minimization of immunogenicity of therapeutic protein products is best accomplished by ensuring stability of the native protein conformation and minimizing formation of high MW species. Our model, thus, envisions that antibody responses to high MW protein aggregates may be elicited in a similar fashion to those elicited by microbial pathogens, (ie, via multivalent ligand cross-linking of BCR).

There are 2 principal pathways by which multivalent ligand cross-linking of BCR enhances immune responses: (1) acceleration of antigen processing and presentation to Th cells,[11] and (2) activation of Bruton's tyrosine kinases mediating B-cell proliferation.[4] Thus, early and rapid IgM responses may be generated to aggregated protein in advance of the provision of T-cell help, but unlike polysaccharide antigens, which are not capable of recruiting T-cell help, protein aggregate-mediated BCR cross-linking would be expected to accelerate the recruitment of T-helper cells.

Induction of Immune Responses by Protein Aggregates: Qualitative Factors

Both qualitative and quantitative factors pertaining to the aggregate species per se, as well as factors independent of protein structure or amount are important in the ability of

any particular aggregate species to induce an immune response. Among the qualitative factors critical in inducing antibody responses are MW and solubility. While low MW aggregates such as dimers and trimers appear inefficient in inducing immune responses, large multimers whose MW exceeds 100 kD are efficient inducers of immune responses. However, multimerization is key to immunogenicity, as larger-sized monomeric proteins are not necessarily more immunogenic than smaller monomeric proteins. Moreover, it has been long known that particulate (insoluble) antigens are very rapidly endocytosed by antigen-presenting cells, which initiate immune responses.[12] More recent work has shown that blood-borne dendritic cells ($CD11c^{lo}$ Mac1+) capture particulate antigens in the periphery, migrate into spleen, and induce TI antibody responses by activating and enhancing the survival of marginal zone (MZ) and B1 B cells.[13] Thus, it is possible that particulate protein aggregates introduced into the systemic circulation meet a similar fate, and traffic to MZ B cells, where the immune response proceeds independent of T-cell help.

Other critical factors bearing on immunogenicity of aggregates pertain to both product and host: product origin (foreign versus endogenous), the presence of product contaminants with immunomodulatory activity, the presence of neoepitopes (as may be created in fusion proteins), and glycosylation/pegylation may exert profound influences on either the formation of aggregates, or the generation of immune responses to aggregates. Host and protocol factors important in determining the potency of aggregate species in triggering immune responses include the frequency of administration, the route of administration, the immunomodulatory activity of the product itself, the host immune status, the activity of concomitant immunomodulators, and for therapeutic versions of endogenous proteins, the robustness of immunologic tolerance to the endogenous protein.

Thus, small amounts of aggregates would be expected to generate a robust response to foreign proteins, but not necessarily for therapeutic counterparts of endogenous proteins to which the immune system has been tolerized. However, the mammalian immune system is not equally tolerant to all endogenous proteins. Most notably, the works of Weigle[14] and of Goodnow[15] have demonstrated that the relative abundance and manner of presentation of the endogenous protein are essential in determining the extent to which the immune system is tolerized to that protein. Moreover, T cells are tolerized more readily by proteins at low abundance than are B cells, perhaps because of the active expression of tissue-specific antigens in the thymus by promiscuous gene expression.[16] Thus, aggregates of relatively low abundance proteins, to which we are not robustly tolerant, might potently trigger immune responses, whereas aggregates of high abundance proteins may have a limited ability to induce immune responses.

Risk Analysis of Protein Aggregates in Therapeutic Protein Products

Protein aggregates pose risk in terms of generation of immune responses to the therapeutic protein product. Of principal concern are those immune responses associated with adverse clinical effects: neutralizing antibody that inhibits the efficacy of the product or, worse, cross-reactively neutralizes an endogenous protein counterpart; and severe immediate hypersensitivity responses such as anaphylaxis.

Regarding immediate hypersensitivity responses, mediated by IgE, the role of protein aggregates in generating such responses is not known. In fact, speculation by Aalberse and Platts-Mills[17] is that aggregates, in increasing the "strength" of an immune stimulant, might deviate an IgE response to a far less devastating IgG4 response. Moreover, a response may be driven from IgE to IgG via TLR-mediated signaling.[18] Nonetheless, once an IgE response has been generated, aggregated allergen is highly efficient at triggering degranulation of mast cells via aggregating IgE receptors.

In considering the mechanism by which protein aggregates might generate neutralizing antibody responses in a highly efficient manner, it is clear that preservation of the conformation of the active site of the protein or proteins within the aggregate is required. This strategy is potentially useful for vaccines, where it is critical to neutralize the natively conformed structures responsible for invasion or toxicity. In contrast, this configuration might prove devastating for therapeutic proteins. Of great importance for avoiding the formation of natively conformed aggregates in therapeutic protein products is product formulation.[19-22] Thus, "structural" formulations that form a bilayer into which proteins can potentially insert, such as nonionic and ionic detergents and liposomes, are of interest. Insertion of proteins into such bilayers is feasible for membrane proteins, which contain clustered hydrophobic residues, whereas such a configuration is not common for soluble therapeutic proteins. Moreover, micelles of the nonionic detergent, polysorbate, are relatively small and likely not capable of incorporating more than a few therapeutic protein molecules, whereas the extended bilayers of ionic detergents, such as sodium dodecylsulfate (SDS), may contain substantial numbers of proteins capable of insinuating themselves into the structure.

More likely to be present in therapeutic protein products are denatured protein aggregates that are generated via unfolding of the native conformation of the protein. While aggregates of denatured protein may potently induce antibody, they would not be expected to induce neutralizing antibody because protein conformation was lost on denaturation. However, antibodies directed to linear epitopes within the

binding site could effectively neutralize if they bound to a linear determinant critical in ligand-receptor binding. Such occurrences appear to be rare. Moreover, through epitope spreading, antibodies that bind to the protein but do not neutralize its activity may facilitate the generation of neutralizing antibodies. However, this is not a rapid and highly efficient process. Thus, for many therapeutic protein products, development of neutralizing antibody is preceded by the existence of a high titered and prolonged binding antibody response. Moreover, other immunologically provocative factors may also be required. In such cases preventing a neutralizing antibody response may be possible by limiting the titer and duration of the primary response, which may limit epitope spread.

Although antibodies that bind but fail to neutralize product are of somewhat lesser concern than neutralizing antibody, in some circumstances such antibodies have been shown to (1) affect the pharmacokinetics of product, necessitating more frequent dosing,[23] (2) mediate anaphylactoid reactions,[24] and (3) potentially facilitate epitope spreading.

Evidence for the Role of Product Aggregates in Induction of Immune Responses to Therapeutic Protein Product: Animal Models

Much of the information framing our understanding of the immunogenicity of protein aggregates and the relative tolerogenicity of soluble protein monomers comes from studies performed in the 1950s and 1960s in which human immunoglobulin (Ig) products were injected into experimental rodents. Such studies found that immune responses to human Ig preparations could be eliminated by techniques that removed high MW material from the preparation.[25] In Gamble's studies in 1966, aggregates generated by heat treatment and added back to deaggregated tolerogenic Ig preparations were shown to induce an immune response that, in a dose-dependent fashion, decreased the time to appearance of antibody and increased the antibody titer.[26]

The use of Ig as a model antigen for studying the effects of aggregates on the immune response was, in retrospect, an interesting one, as these proteins differ from most other proteins in terms of complex immune system interactions. Thus, Ig can modify activation of the immune response to itself through the binding and coligation of multiple receptors on the B-cell surface. While the antigen binding fragment (Fab) of an antibody molecule binds to BCR, the complement fixing fragment, Fc, can simultaneously bind to Fc receptors on the B-cell surface. Coligation of BCR with FcR (FcγRII), the only (and inhibitory) FcR expressed on B cells, limits antibody responses.[27] In contrast, complement bound to the Fc moiety of an antibody molecule can bind to complement receptors (CR2) on the B-cell surface. In this case, simultaneous ligation of BCR and complement receptor (CR2) on the B-cell surface profoundly enhances antibody production.[27] Aggregated IgG and pentameric IgM bound to antigen can directly fix and activate complement. Whether such factors influenced immunogenicity of the aggregated species in these studies in mice is not certain, as the affinity and binding of human Ig with rodent complement, complement receptors, and FcRs is not clear.

Although ordinary protein antigens cannot simultaneously bind to BCR and FcR or CR, immune responses to proteins may be potentiated by the formation of protein:antibody complexes. For example, IgM potently enhances antibody responses to particulate antigen, while IgG enhances responses to soluble proteins. Reciprocally, IgM does not enhance responses to small soluble proteins and IgG suppresses responses to particulate antigens. The boost in antibody response resulting from the binding of IgM to antigen requires complement fixation and activation, whereas enhancement through IgG is mediated by engagement of activating FcR on non-B cell antigen presenting cells, such as macrophages.[27] Thus, IgM bound to large protein aggregates may well enhance the antibody response through direct uptake by complement receptors, or via the simultaneous ligation of BCR by the aggregated protein and CR through complement fragments bound to Fc. IgG binding to smaller soluble aggregates may theoretically also enhance antibody responses via activating FcR.

The ability of protein aggregates to elicit antibody responses to a self-protein was most objectively and thoroughly evaluated by Braun et al, who employed human interferon alpha (huIFN-α) transgenic mice to evaluate the capacity of IFN-α product aggregates to break tolerance.[28] They clearly demonstrated that homogenous aggregates of huIFN-α and composite aggregates of mouse serum albumin and huIFN-α potently induced antibody to huIFN-α, whereas IFN-α monomer failed to do so (within the time frame of the study). Unfortunately, for the preparation containing homogenous huIFN-α aggregates, the characteristics of the aggregate species responsible for the efficiency of response induction—the large amount of dimer in the product versus the "very small amount" of high MW aggregate—were not explored. Also critical in this study was the elucidation of the role of dosing frequency, route of administration, and immunomodulation on generation of antibodies, albeit in response to nonaggregated IFN-α monomer. However, these factors may also be critical in the ability of protein aggregates to trigger immune responses, particularly to antigens to which the immune system is tolerant. Thus, more frequent dosing, a subcutaneous (SC) or intraperitoneal (IP) route of administration rather than an intravenous (IV) route, and administration of concomitant immunomodulators would be expected to facilitate the ability of small amounts of aggregates to enhance the immune response.

Evidence for the Role of Product Aggregates in Induction of Immune Responses to Therapeutic Protein Product: Clinical Studies

Clinically, evidence that aggregates in therapeutic protein products had profound effects on immunogenicity was apparent from very early studies. In the 1950s and early 1960s intravenous immune globulin (IVIG) preparations contained substantial aggregated material that triggered severe hypersensitivity (anaphylactoid) responses due to fixation and activation of the complement cascade and also potentially to release of histamine.[29,30] Antibody responses to such aggregates appeared to be specific for novel determinants present only on the aggregated species (ie, for higher order structures) or for normally cryptic determinants, which are exposed on aggregates of denatured protein.[26] Of importance, responses were far more prevalent and potent in the antibody-deficient patient population than in those without antibody deficiency, suggesting that antibodies generated to human Ig in nontolerant patients increased the frequency and severity of these reactions.[29] These types of adverse clinical responses were also noted in patients treated with early commercial preparations of human serum albumin (HSA) and pasteurized plasma solutions.[24]

A further example of the potency of large amounts of aggregates on generation of immune responses is that of the clinical experience with human growth hormone (hGH). Originally purified from formalin-fixed pituitary glands, hGH contained substantial amounts of aggregates (50%-70%) that induced immune responses. Although hGH prepared by an improved method contained substantially less aggregated material (10%), antibody responses were nevertheless still substantial. Intriguingly, the level of aggregates appeared to determine not whether there would be an antibody response, but rather the nature of the antibody response. Patients treated with the heavily aggregated product demonstrated persistent antibodies to hGH, whereas those treated with the lesser aggregated product developed a transient antibody response.[31] Despite the frequency of appearance of binding antibody in response to hGH therapy, the development of neutralizing antibody was uncommon. However, neutralizing antibody occurred much more commonly in patients with severe congenital isolated GH deficiency (ie, in protein "knock out" children in whom the lack of hGH protein conferred a lack of immune tolerance to the hormone).

The therapeutic protein IL-2 can be viewed as a good test of the general principles governing immune response generation to large MW aggregates expressing multivalent antigens. Indeed, although the monomeric protein is ~15 kD, the recombinant human product is formulated as an aggregate with an average size of 27 molecules per aggregate.[32] Thus, a considerable amount of the product has a MW >100 kD as well as having >20 ligands per aggregate, conditions that were shown to optimize immunogenicity. Indeed, this product is highly immunogenic, inducing binding antibody responses in 80% to 100% of patients.[33] Of interest, neutralizing antibody to IL-2 largely arises in a group of patients treated with IL-2 in an immunologically provocative fashion (ie, SC administration or concomitant treatment with IFN-α, a known immunomodulator, and extensive treatment over a prolonged time period).[33] Also intriguing is the time course in which the neutralizing response arises, as it is detected several months following detection of binding antibody, implicating epitope spreading as the likely route of development. Moreover, it is not clear whether T-cell help is required for either binding or neutralizing antibody.

In fact, these few examples illustrate a more general principle, which is that for most therapeutic proteins, product neutralizing antibody is much less common than product binding antibody. Neutralizing antibody arises more frequently with the following risk factors: generation of a high titer binding antibody response, sustained treatment, and treatment by a more immunogenic route (ie, SC), concomitant treatment with immune modulators, and genetic deficiency of the factor. There are some very notable exceptions to this rule, one being the case of immune responses to the TPO congener, pegylated megakaryocyte growth and development factor (PEG-MGDF), in which as few as 2 doses were sufficient to induce a vigorous neutralizing antibody response.[9] However, the very low abundance of the endogenous protein counterpart, TPO, in normal physiology[34] predicts that tolerance to this protein would be minimal, a situation somewhat akin to individuals with genetic deficiencies of particular proteins (such as hGH) who are highly prone to formation of neutralizing antibody. The SC route of administration was an additional provocative factor in triggering the TPO neutralizing response, as was the immune competency of the patient population. Moreover, whether immunogenicity was triggered by truncation of the full length TPO, thereby exposing the normally protected active site to the immune system, is not clear. Certainly the pegylation of the truncated TPO would have been expected to confer protection against immune response generation.

Why the immune system commonly makes robust responses to nonneutralizing determinants yet has to be mightily provoked to make responses to neutralizing determinants, suggests that the immune system is more tolerant (or ignorant) of the active site than of other epitopes, which raises several interesting possibilities: the active sites of self-proteins are normally very well shielded from antigen specific T- and/or B-cells[35]; the active sites of proteins lack primary sequence for complexation with self Class II MHC and thus fail to be T-cell immunogens; or the active sites of proteins may be exposed to the immune system in such a way as to induce a

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