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In Vivo Effect of Innate Immune Response Modulating Impurities on the Skin Milieu Using a Macaque Model: Impact on Product Immunogenicity

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ABSTRACT

Unwanted immune responses to therapeutic proteins can severely impact their safety and efficacy. Studies show that the presence of trace amounts of host cells and process-related impurities that stimulate pattern recognition receptors (PRR) can cause local inflammation and enhance product immunogenicity. Here we used purified PRR agonists as model impurities to assess the minimal level of individual innate immune response modulating impurities (IIRMIs) that could activate a local immune response. We show that levels of endotoxin as low as 10 pg (0.01 EU), 1 ng for polyinosinic:polycytidylic acid (PolyI:C), 100 ng for synthetic diacylated liopprotein, thiazoloquinolone compound, or muramyl dipeptide, 1 μg for flagellin or β-glucan, or 5 μg for CpG-oligodeoxynucleotide increased expression of genes linked to innate immune activation and inflammatory processes in the skin of rhesus macaques. Furthermore, spiking studies using rasburicase as a model therapeutic showed that the levels of PRR agonists that induced detectable gene upregulation in the skin were associated with increased immunogenicity for rasburicase. This study underscores the need for testing multiple IIRMIs in biologics, strengthening the connection between the local mRNA induction in skin, innate immune activation, and antibody development in primates, and provides an indication of the levels of IIRMI in therapeutic products that could impact product immunogenicity.
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Introduction

Immune responses to therapeutic products are frequent and can significantly affect their safety and efficacy.[1,2] The consequences of these are exemplified by the episodes of pure red cell aplasia that followed the development of antibodies to EPREX®, or the reduced therapeutic efficacy subsequent to the development of antibodies to α-glucosidase, IFNβ (interferon β), or coagulation factor VIII.[3,4] Although all proteins, self and foreign, bear antigenic sites to which an immune response can theoretically be directed, the actual development of an immune response is thought to depend on numerous product-related (homology to self, post-translational modifications, presence of aggregates), and patient-related (e.g., age, treatment regimen, immune status) factors.[5,6] Understanding and controlling the factors that promote immunogenicity to therapeutic products is critical to obtaining safe and effective products and may reduce the need for extensive clinical trials to assess immunogenicity.[7] One of the elements that may increase the risk of immunogenicity of a product is the presence of host cell-derived and process-related impurities that can stimulate innate immune receptors in the host by acting as adjuvants.

Biotechnology products, whether they are recombinant or naturally derived, are manufactured using complex expression and production systems that usually involve genetically modified bacteria, yeast, insect or mammalian cells, and fermentation media.[5,8] Downstream purification processes are designed to eliminate impurities such as residual host DNA, host cell proteins, and endotoxins; however, trace levels of these may copurify with the product. For example, although parenteral products are sterile, they may have detectable bioburden until the latest stages of drug substance manufacturing.[9] As a result, trace amounts of host cell or process-derived impurities could be deposited together with the product in the tissue and foster an inflammatory or immune response. The cells in the subcutaneous (SC) tissue and peripheral blood express a variety of surface pattern recognition receptors (PRR). Examples of PRRs include transmembrane Toll-like receptor (TLR) and C-type lectin receptor, as well as a variety of cytoplasmic receptors that bind nucleic acids or peptidoglycans (e.g., bacterial

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muramyl dipeptide) expressed on stromal (particularly in epithelial cells in skin and other barrier tissues) and immune cells (such as macrophages and dendritic cells [DCs]).[10] Each of these receptors are triggered by various highly conserved microbial structures (which we designate PRR agonists [PRRAgs]), including bacterial carbohydrates (such as lipopolysaccharide [LPS] and mannose), nucleic acids (DNA or RNA), bacterial peptides (flagellin, microtubule elongation factors), peptidoglycans and lipoteichoic acids, lipoproteins, fungal glucans, and chitin.[11] In addition, these receptors are also activated by molecular patterns associated with cellular stress and tissue damage (e.g., heat shock proteins or reactive oxygen species).[12] Activated PRR trigger several intracellular signaling pathways leading to the production of proinflammatory cytokines (interleukin-6 [IL-6]), tumor necrosis factorα, interferons [IFNs]) and chemokines (CXCL8/IL-8, CCL5, CXCL10) that attract granulocytes, as well as macrophages, DCs, and other antigen presenting cells (APCs) to the site, which enhance their antigen-presenting capabilities, foster trafficking to the local lymph nodes, and enable the initiation of an immune response.[13-16] When impurities can activate PRR, we designate them as innate immune response modulating impurities (IIRMIs).

The slow absorption, ease of self-administration, and the development of auto-injectors have made the SC tissue the most popular parenteral route for biologics. This route has been associated with higher immunogenicity risk for some products and this could be linked to the innate immune system in the skin.[17] The major population of cells in the skin stroma—keratinocytes, fibroblasts, and melanocytes—express a wide variety of PRRs.[18-20] For example, human keratinocytes express most TLR although some may be nonfunctional; melanocytes express TLR4; and fibroblasts express TLR3 and TLR4.[14,21-24] Importantly, the skin and SC space are also populated with resident and trafficking immune cells including Langerhans cells, monocytes/macrophages, DCs, innate lymphoid cells, lymphocytes, and mast cells, each expressing an array of PRRs including TLRs, dectin1, and nucleotide-binding oligomerization domain-like receptors that can initiate and instruct a local innate immune and inflammatory cell, as well as foster a systemic immune response.[19,20] Among the DCs that infiltrate the skin and SC tissue, TLRs 1, 2, 3, 4, 5, 6, 8, and 10 are usually expressed in myeloid DCs (mDCs), Langerhans cells express TLR1, 2, 3, 5, 6, whereas plasmacytoid DCs, which express TLRs 7 and 9, do not reside in the skin but traffic to it upon stimulation. Thus, for example, remnants of a bacterial cell wall may activate mDCs via TLR2 and TLR4 leading to the activation of nuclear factor kappa-light chain (NF-kB), whereas nucleic acids may trigger TLRs 3 and 7 on mDC and plasmacytoid DC eliciting a downstream response mediated by IRF3 and IRF7.[25] Currently, there are readily available validated assays to detect few impurities such as endotoxins and DNA and these are regularly monitored in biotechnology products; however, other impurities that can act as adjuvants such as flagellin or β-glucans are seldom monitored. In previous studies, our group and others have shown that very low levels of PRRAgs are sufficient to foster the secretion of cytokines and immunoglobulins *in vitro*, and that impurities may synergize in their adjuvant effect.[14,26-28] While IIRMIs were shown to increase immunogenicity in mice, an understanding of the minimal amounts of these PRRAgs that could increase the immunogenicity risk of a product in humans is lacking. Because the *in vitro* response of nonhuman primates to IIRMI appears to be similar to that of humans, we have used nonhuman primates to gain some understanding of the levels of these impurities that can activate a local inflammatory or innate immune response *in vivo*.

Most of the studies *in vivo* assessing the factors involved in product immunogenicity have been conducted in mice. To assess the effect of IIRMI, the differences in the cellular distribution of PRRs and the architecture of the skin hinder the translation of the findings in mice to human.[29] Indeed, humans have 10 TLRs and 22 NLRs, whereas mice have 12 and 34 respectively,[30-33] and TLR8, which responds to single-stranded RNA, in human and nonhuman primates is not functional in mice.[34] Preclinical studies are often conducted in nonhuman primates. Although there is some variation in PRR expression between Rhesus and humans, they exhibit a more comparable cellular distribution and expression pattern of TLRs on APCs and other cell types than mice, and more similar skin architecture to that of humans.[35] In addition, the response *in vitro* to TLR agonists appears to be conserved between humans and rhesus macaques.[36,37] This makes them a good model for understanding the potential impact of IIRMI on product immunogenicity.[38,39] In this study, we used the macaque model to explore whether IIRMI can act as adjuvants, increasing the immunogenicity risk of therapeutic proteins administered subcutaneously. We show that trace levels of PRRAgs can elicit detectable immune response in the skin of nonhuman primates marked by increased expression of mRNA for chemokines, cytokines, and cell surface receptors linked to antigen presentation. This suggests that these PRRAgs could increase antigen uptake and presentation by local immune cells leading to enhanced T and B cell responses.[40] Finally, we show that coadministration of a foreign protein with the levels of PRRAgs that induce a local response is associated with increased anti-drug antibody (ADA) levels to the protein in primates. This is important as several studies in human patients show that higher antibody titers are correlated with clinical impact.[41]

Materials and Methods

Reagents

Antigens binding PPRAgs are as follows: LPS from *Salmonella minnesota* Re595 was purchased from Calbiochem (Darmstadt, Germany), polyinosinic:polycytidylic acid (PolyI:C), flagellin, synthetic diacylated liopprotein (FSL-1), imiquimod, MDP, and β-glucan were purchased from InvivoGen (San Diego, CA). CpG-oligodeoxynucleotide (ODN) D35 (D-ODN; 5′-ggTGCATCGATGCAGGGGgg-3′) was synthesized at the FDA core facility (Rockville, MD).[37] PRRAgs were used at the concentration indicated in each individual figure. The purity of the individual IIRMI was confirmed using human embryonic kidney 293 cell expressing individual PRR. Rasburicase (Elitek®) is a recombinant urate oxidase indicated for initial management of plasma uric acid levels in pediatric and adult patients with leukemia, lymphoma, and solid tumor malignancies who are receiving anticancer therapy expected to result in tumor lysis and subsequent elevation of plasma uric acid.[42]

Animals

Twenty-five male and female rhesus macaques (*Macaca mulatta*) were maintained at the FDA White Oak animal facility. All experiments were approved by the White Oak Consolidated Animal Care and Use Committee, and conducted in accordance with recommendations put forth in the Guide for the Care and Use of Laboratory Animals, eighth edition. Macaques were housed in approved facilities and monitored daily by veterinary staff. Nonhuman primate groups were weight and gender matched (n = 4-5 per group).

For the SC injection, animals were anesthetized with ketamine (5-10 mg/kg) prior to inoculation so that they were safely immobilized. Skin biopsies were taken 6 h later under ketamine (5-10 mg/kg) and xylazine (0.5-1 mg/kg).

TLR Agonist Treatment

PRRAgs were prepared in 50 μL of sterile saline and injected subcutaneously using a 30-gauge needle in the shaved chest.