

Guidance for Industry Immunogenicity Assessment for Therapeutic Protein Products

DRAFT GUIDANCE

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**U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)**

**February 2013
Clinical/Medical**

Guidance for Industry

Immunogenicity Assessment for

Therapeutic Protein Products

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38 FDA's guidance documents, including this guidance, do not establish legally enforceable
39 responsibilities. Instead, guidances describe the Agency's current thinking on a topic and
40 should be viewed as recommendations, unless specific regulatory or statutory requirements are
41 cited. The use of the word *should* in Agency guidances means that something is suggested or
42 recommended, but not required.

43

44

II. BACKGROUND

46

47 Immune responses to therapeutic protein products may pose problems for both patient safety
48 and product efficacy. Immunologically based adverse events, such as anaphylaxis, cytokine
49 release syndrome, so-called “infusion reactions,” and nonacute immune reactions such as
50 immune complex disease (see Appendix C), have caused sponsors to terminate the development
51 of therapeutic protein products or limited the use of otherwise effective therapies. Unwanted
52 immune responses to therapeutic proteins may also neutralize the biological activity of
53 therapeutic proteins and may result in adverse events not only by inhibiting the efficacy of the
54 therapeutic protein product, but by cross-reacting to an endogenous protein counterpart, if
55 present (e.g., neutralizing antibodies to therapeutic erythropoietin may cause pure red cell
56 aplasia by also neutralizing the endogenous protein) (Murphy 2011; Worobec and Rosenberg
57 2004; Rosenberg and Worobec 2004; Rosenberg and Worobec 2005; Koren, et al. 2008;
58 Hermeling, et al. 2004). Because most of the adverse effects resulting from elicitation of an
59 immune response to a therapeutic protein product appear to be mediated by humoral
60 mechanisms, circulating antibody (to the therapeutic protein product) has been the chief
61 criterion for defining an immune response to this class of products.³

62

63 Both patient-related and product-related factors may affect immunogenicity of therapeutic
64 protein products. These factors provide the starting point for an immunogenicity risk
65 assessment. Ideally, these factors should be taken into consideration in the early stages of
66 therapeutic protein product development. Below is a more detailed discussion of the nature of,
67 and risk factors for, the more common immune responses to therapeutic protein products as
68 well as possible mitigation strategies that may be employed.

69

70

III. CLINICAL CONSEQUENCES

72

73 Treatment of patients with therapeutic protein products frequently results in immune responses
74 of varying clinical relevance, ranging from transient antibody responses with no apparent
75 clinical manifestations to life-threatening and catastrophic reactions. During therapeutic protein
76 product development, elucidation of a specific underlying immunologic mechanism for related
77 adverse events is encouraged, because this information can facilitate the development of
78 strategies to help mitigate the risk of clinically significant immune responses. The extent of
79 information required to perform a risk-benefit assessment will vary among individual products,

³ IgG and IgE antibody responses are those most often associated with clinical adverse events and their generation generally requires collaboration between antigen-specific T helper cells and B cells (Murphy 2011).

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80 depending on product origin and features, the immune responses of concern, the target disease
81 indication, and the proposed patient population.

82

A. Consequences for Efficacy

84

85 Development of both neutralizing and non-neutralizing antibodies can limit product efficacy in
86 patients treated with therapeutic protein products. Neutralizing antibody can block the efficacy
87 of the product, which is of utmost concern if the product is a life-saving therapeutic. Even if
88 not in the context of a life-saving therapeutic, loss of efficacy can be problematic. Neutralizing
89 antibody that cross-reacts with a nonredundant endogenous counterpart can also impact safety,
90 as discussed in the next section. Non-neutralizing (binding) antibody may alter the
91 pharmacokinetics of the product, by either diminishing or enhancing product pharmacokinetic
92 parameters, and therefore may require dosing modifications (Wang, et al. 2008). However, if
93 present at high enough titer, non-neutralizing antibody may also mistarget the therapeutic
94 protein into Fc Receptor (FcR) bearing cells, thereby reducing product efficacy (Wang, et al.
95 2008). Furthermore, although some binding antibodies may have no apparent effect on clinical
96 safety or efficacy, they may promote the generation of neutralizing antibodies via the
97 mechanism of epitope spreading (Disis, et al. 2004). Correlation with clinical responses is
98 usually necessary to determine the clinical relevance of both binding and neutralizing antibody
99 responses.

100

B. Consequences for Safety

102

103 The safety consequences of immunogenicity may vary widely and are often unpredictable in
104 patients administered therapeutic protein products. Therefore, a high index of suspicion for
105 clinical events that may originate from such responses should be maintained, even if the initial
106 risk assessment suggests a lower risk of immunogenicity. The following section describes a
107 few of the major safety concerns associated with immunogenicity.

108

1. Anaphylaxis

110

111 Anaphylaxis is a serious, acute allergic reaction characterized by certain clinical features. The
112 definition currently accepted by the Agency relies on clinical diagnostic criteria and does not
113 specify a particular immunologic mechanism (Sampson, et al. 2006 and see Appendix).
114 Historically, the definition of anaphylaxis has invoked the involvement of specific IgE
115 antibodies. However, such a mechanistic definition is problematic in the context of therapeutic
116 protein product development and other clinical settings where it may not be possible to identify
117 a specific immunologic mechanism as the basis of an adverse event. In the interest of capturing
118 all potential adverse events of interest, the Agency recommends identifying all cases meeting
119 the clinical diagnostic criteria of anaphylaxis, regardless of the presumed pathophysiology.
120 Additional information, such as the detection of elevated serum histamine or tryptase levels
121 following a reaction or product-specific IgE antibodies may help elucidate the pathophysiology
122 of the anaphylactic response.

123

124 Furthermore, the presence of anti-product antibody alone is not necessarily predictive of
125 anaphylaxis or other hypersensitivity reactions. Correlation with clinical responses is typically

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126 required to determine the clinical relevance of these antibodies. Determination of the
127 underlying mechanism remains of interest, however, since anaphylaxis with confirmation of
128 IgE involvement has certain prognostic implications for repeat exposure, as well as for potential
129 therapeutic options for mitigation.

130

131 2. *Cytokine Release Syndrome*

132

133 Cytokine release syndrome is a symptom complex caused by the rapid release of
134 proinflammatory cytokines from target immune cells (Stebbing, et al. 2007). The underlying
135 mechanism is not fully understood, and multiple mechanisms, such as binding of activating Fc
136 Receptors and clustering of the antigen on target cells, may be involved for different products.
137 Pre- and post-dose cytokine levels may provide evidence to support the clinical diagnosis and
138 distinguish the symptom complex from other acute drug reactions (see Appendix).

139

140 3. *“Infusion Reactions”*

141

142 Therapeutic proteins may elicit a range of acute effects, from symptomatic discomfort to
143 sudden, fatal reactions that have often been grouped as “infusion reactions” in the past (see
144 Appendices A and B). Although the term implies a certain temporal relationship, infusion
145 reactions are otherwise not well defined and may encompass a wide range of clinical events,
146 including anaphylaxis and cytokine release syndrome. In the absence of an agreed-upon
147 definition for “infusion reaction,” the categorization of certain adverse events as infusion
148 reactions without further detail is problematic and is not recommended. Sponsors are
149 encouraged to use more descriptive terminology when possible, noting the timing, duration, and
150 specific signs and symptoms observed upon administration of a therapeutic protein. Data from
151 mechanistic studies may be able to discriminate specific antibody-mediated anaphylaxis from
152 episodes pertaining to cytokine release phenomena.

153

154 4. *Non-acute Reactions*

155

156 Anaphylaxis, cytokine release syndrome, and other acute reactions are temporally linked to
157 administration of a therapeutic protein product. Delayed hypersensitivity and immune
158 responses secondary to immune complex formation typically have a subacute presentation. As
159 a result, the association between a therapeutic protein product and these reactions may be more
160 difficult to establish, and confirmation of the underlying mechanism may not be easily
161 achieved. Clinical signs may include delayed onset of fever, rash, arthralgia, myalgia,
162 hematuria, proteinuria, serositis, central nervous system complications, and hemolytic anemia
163 (Hunley, et al. 2004; Goto, et al. 2009). When such a reaction is suspected, laboratory
164 assessment for circulating immune complexes may help confirm the diagnosis.

165

166 5. *Cross-reactivity to Endogenous Proteins*

167

168 Anti-drug antibody can have severe consequences if it cross-reacts with and inhibits a non-
169 redundant endogenous counterpart of the therapeutic protein product or related proteins. If the
170 endogenous protein is redundant in biological function, inhibition of the therapeutic and
171 endogenous proteins may not produce an obvious clinical syndrome until the system is stressed,

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172 because not all biological functions of an endogenous protein may be known or fully
173 characterized. Moreover, the long-term consequences of such antibodies may not be known.

174

175 For therapeutic protein counterparts of endogenous proteins that are critical to normal fetal or
176 neonatal development, neutralization of such endogenous proteins, resulting from antibodies to
177 the therapeutic protein counterpart may potentially negatively impact fetal or neonatal
178 development when such responses are generated during pregnancy or breast feeding. Indeed,
179 the potential transmission of antibodies to developing neonates by breast milk must be
180 considered. Therefore, the risk of neutralizing antibody development following administration
181 of such therapeutic proteins to women of childbearing potential should be strongly considered
182 in light of their potential benefit.

183

184

185 IV. RECOMMENDATIONS FOR IMMUNOGENICITY RISK MITIGATION IN 186 THE CLINICAL PHASE OF DEVELOPMENT OF THERAPEUTIC PROTEIN 187 PRODUCTS

188

189 Given the variety of factors that can affect immunogenicity, the risk assessment and appropriate
190 mitigation strategies will depend on the individual development program and should be
191 considered at an early stage and at each stage of product development. The extent of
192 immunogenicity safety information required premarketing and postmarketing will vary,
193 depending on the potential severity of consequences of these immune responses and the
194 likelihood of their occurrence.

195

196 In terms of evaluating the clinical relevance of immune responses, the Agency has the
197 following recommendations:

198

199 Assay development

200

- 201 • Assay development is covered in detail in draft guidance (see Draft Guidance for
202 Industry entitled “*Assay Development for Immunogenicity Testing of Therapeutic
203 Proteins*”). Sponsors should develop and implement sensitive, qualified
204 immunoassays commensurate with the overall product development program.
205 Concomitant sampling of therapeutic product levels is recommended to assess
206 potential interference with the assay.

207

208 Product-specific antibody sampling considerations

209

- 210 • Baseline serum samples for anti-product antibody testing should be collected, and
211 sampling frequency and duration should reflect anticipated use of the product. More
212 frequent sampling is appropriate during the initiation and early use of a new,
213 chronically administered product; less frequent sampling may be appropriate after
214 prolonged use. Repeat sampling should generally occur over periods of sufficient
215 duration to determine whether antibody responses are transient, whether a
216 neutralizing antibody response has developed, and whether these responses are
217 associated with long-term clinical sequelae.

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- In addition to a prespecified sampling schedule, unscheduled sampling triggered by suspected immune-related adverse events is useful for establishing the clinical relevance of antiproduct antibodies.

223

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225

Dosing

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- Banking of serum samples from clinical trials under appropriate storage conditions for future testing is always advisable.
- For first-in-human trials, a conservative approach in an appropriate medical setting with staggered dosing among individual patients, dosing cohorts, and different routes of administration is generally appropriate. The trial design should include prespecified dose escalation criteria and adequate time intervals between dosage cohorts and, as appropriate for the pharmacokinetics and pharmacodynamics of the product, between individuals within a dose cohort to assess toxicities prior to administration of subsequent doses or treatment of additional individuals. The need for such an approach will depend on the individual circumstances. As development progresses, dosing strategies and safety parameters can be modified based on clinical experience with the product and related products.

238

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- Because predicting the effects of product-specific antibodies may be difficult during therapeutic protein product development, dosing regimens in subsequent studies should be risk based, taking into account the data from initial trials, the potential for cross-reactivity to endogenous proteins or neutralization of the therapeutic protein product, clinical parameters that impact immunogenicity in different patient populations, and the adequacy of the proposed safety monitoring.

245

Adverse events

246

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- The development of neutralizing antibody activity or the presence of sustained, high antibody titers may lead to loss of efficacy or an increased risk of an adverse reaction. In certain situations (e.g., assessment of a product with a nonredundant endogenous counterpart), real-time assessments for antibodies during a clinical trial may be recommended for safety reasons. The need for such intensive monitoring will depend on the individual circumstances.

254

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259

- If clinically relevant immune responses are observed, sponsors are encouraged to study the underlying mechanism and identify any critical contributing factors. These investigations can facilitate development of potential mitigation strategies, including modification of product formulation, screening of higher-risk patients, or adoption of risk mitigation strategies (see below).

260

261

262

- In some cases, sponsors may choose to explore desensitization or immune tolerance induction procedures as potential mitigation strategies. Given the risks associated with desensitization/immune tolerance induction procedures, the appropriateness of

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263 such investigations will depend on the nature of the specific indication, the target
264 patient population, and the stage of development.

265
266 Comparative immunogenicity studies

- 267
- 268 • For all comparative immunogenicity studies (e.g., those comparing antibody
269 incidence, titer, or neutralizing activity to product pre- and post-manufacturing
270 changes), a strong rationale and, when possible, prespecified criteria should be
271 provided to justify what differences in incidence or severity of immune responses
272 would constitute an unacceptable difference in product safety.⁴

273
274 Postmarketing safety monitoring

- 275
- 276 • Robust postmarketing safety monitoring is an important component in ensuring the
277 safety and effectiveness of therapeutic protein products. Because some aspects of
278 postmarketing safety monitoring are product-specific, FDA encourages sponsors to
279 consult with appropriate FDA divisions to discuss the sponsors' proposed approach to
280 postmarketing safety monitoring. Rare, but potentially serious, safety risks (e.g.,
281 immunogenicity) may not be detected during preapproval clinical testing, because the
282 size of the population exposed may not be large enough to assess rare events. In
283 particular cases, such risks may need to be evaluated through postmarketing
284 surveillance or studies.

285
286

287 **V. PATIENT- AND PRODUCT-SPECIFIC FACTORS THAT AFFECT** 288 **IMMUNOGENICITY**

289

290 **A. Patient-Specific Factors That Affect Immunogenicity**

291

292 Factors related to the target patient population may increase or decrease the risk of an immune
293 response. Therefore, caution is recommended when moving from one patient population to
294 another.

295

296 *1. Immunologic Status and Competence of the Patient*

297

298 Patients who are immune suppressed may be at lower risk of mounting immune responses to
299 therapeutic protein products compared to healthy volunteers with intact immune responses. For
300 example, 95 percent of immune-competent cancer patients generated neutralizing antibody to a
301 Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) product, but only 10 percent of
302 immune-compromised cancer patients did so (Ragnhammar, et al. 1994). Immune suppression
303 with agents that kill antigen-activated lymphocytes and/or elicit activity of regulatory T cells,
304 such as methotrexate, can have a substantial effect on immunogenicity of co-administered
305 therapeutic protein products (Baert, et al. 2003). In contrast to immune-deficient patients,
306 patients with an activated immune system (e.g., patients with certain infections or autoimmune

⁴ For information on proposed biosimilar products, see draft guidance titled *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product* (February 2012).

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307 disease) may have augmented responses. Immune response generation may also be affected by
308 patient age, particularly at the extremes of the age range. Particular caution should be used in
309 studies evaluating novel therapeutics in healthy volunteers with regard to immunogenicity and
310 immune responses (Stebbins, et al. 2007; Li, et al. 2001).

311

312 *Recommendation*

313

314 In the development of therapeutic protein products, a rationale should be provided to support
315 the selection of an appropriate study population, especially for first-in-human studies.

316

317 2. *Prior Sensitization/History of Allergy*

318

319 Prior exposure to a therapeutic protein or to a structurally similar protein may result in a
320 sensitized patient at baseline. This is a particular concern for patients receiving factor or
321 enzyme replacement therapy, who may have antibodies to a previous replacement product that
322 could cross react on an analogous product.

323

324 Sensitization to the excipients or process/product related impurities of a therapeutic product
325 may also predispose a patient to an adverse clinical consequence. For example, products
326 produced from transgenic sources may contain allergenic foreign proteins, such as milk protein
327 or protein from chicken eggs.

328

329 *Recommendation*

330

331 Screening for a history of relevant allergies is recommended, and the appropriateness of
332 administration will depend on the individual risk-benefit assessment.

333

334 3. *Route of Administration, Dose, and Frequency of Administration*

335

336 Route of administration can affect the risk of sensitization. In general, intradermal,
337 subcutaneous, and inhalational routes of administration are associated with increased
338 immunogenicity compared to the intramuscular and the intravenous (IV) routes. The IV route is
339 generally considered to be the least likely to elicit an immune response. In conjunction with the
340 route of administration, the dose, frequency, and duration of treatment can also affect
341 immunogenicity (Rosenberg and Worobec 2004). For example, a lower dose administered
342 intermittently is typically more immunogenic than a larger dose administered without
343 interruption. It should be noted that the effects of dose and frequency on immune responses to
344 therapeutic protein products are not independent of other factors, such as route of
345 administration, product origin, and product-related factors that influence immunogenicity (see
346 below).

347

348 *Recommendations*

349

350 Immunogenicity should be considered when selecting an appropriate route of administration,
351 especially for high-risk therapeutic protein products (e.g. therapeutic counterparts of
352 nonredundant endogenous proteins) in first-in-human dosing.

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353

354 Changes in the route of administration or dosing during product development may be associated
355 with changes in the immunogenicity profile, and clinical safety data to support such changes are
356 recommended.

357

358

4. Genetic Status

359

360 Genetic factors may modulate the immune response to a therapeutic protein product. In
361 particular, some Human Leukocyte Antigen (HLA) haplotypes may predispose patients to
362 development of undesirable antibody responses to specific products (Hoffmann, et al. 2008). If
363 feasible, HLA mapping studies may help define a subset of the patient population at increased
364 risk. Moreover, genetic polymorphisms in cytokine genes may upregulate or downregulate
365 immune responses (Donnelly, Dickensheets, et al. 2011).

366

Recommendation

367

368

369 Evaluation of genetic factors that may modulate the immune response to a therapeutic protein
370 product is recommended. For example, the subset of patients that generate neutralizing
371 antibodies to IFN-beta products are more likely to possess distinct HLA haplotypes (Hoffmann,
372 et al. 2008). Thus, knowledge of the heightened susceptibility of patients with such HLA
373 haplotypes may allow for measures to prevent such responses or to pursue other treatment
374 options.

375

376

5. Status of Immune Tolerance to Endogenous Protein

377

378 Humans are not equally immunologically tolerant to all endogenous proteins. Thus, the
379 robustness of immune tolerance to an endogenous protein affects the ease with which a
380 therapeutic protein product counterpart of that endogenous protein can break such tolerance.
381 Immunological tolerance in both protein-specific T and B cells depends on many factors,
382 prominent among which is the abundance of the endogenous protein: immune tolerance is
383 weaker for low-abundance and stronger for high-abundance proteins (Weigle 1980; Goodnow
384 1992; Haribhai, et al. 2003).

385

386 The human immune system is not fully tolerant to low-abundance endogenous proteins, such as
387 cytokines and growth factors, for which serum levels may be in the nanogram (ng)/milliter
388 (mL) to picogram (pg)/mL range. This point is underscored by the presence of autoantibodies to
389 cytokines and growth factors in healthy individuals, the development of antibodies to
390 inflammatory cytokines, and the breaking of tolerance to endogenous proteins by administration
391 of exogenous recombinant therapeutic protein products (Worobec and Rosenberg 2004;
392 Rosenberg and Worobec 2004; Rosenberg and Worobec 2005; Koren, et al. 2008; Hermeling,
393 et al. 2004). When a therapeutic protein is intended as a replacement for an absent or deficient
394 endogenous protein, patients with genetic mutations conferring a “knock out” phenotype may
395 respond to the therapeutic product as to a foreign protein or neoantigen, or may already be
396 sensitized as a result of previous exposure to a similar therapeutic protein or related proteins
397 from other sources.

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399 *Recommendations*

400

401 For a therapeutic protein product that is a counterpart of an endogenous protein, particularly if
402 for first-in-human use, and for high-risk therapeutic proteins (e.g., those with endogenous
403 protein counterparts with nonredundant functions), some understanding of the robustness of
404 immune tolerance to that endogenous protein should be gained by the following:

405

406 • Quantitating or gathering information on the level of the endogenous protein in
407 serum in the steady state, as well as in conditions that specifically elicit its
408 production

409

410 • Assessing for, or gathering information on, the presence of pre-existing antibodies in
411 healthy individuals and patient populations

412

413 • Incorporating evaluations of immunogenicity, immune cell activation, inflammatory
414 responses, or cytokine release into relevant animal studies to obtain insight and
415 provide guidance for clinical safety assessments (see Appendix, part E) (Koren
416 2002)

417

418 Consideration should also be given to the following:

419

420 • Evaluation of the genetic status (e.g., cross-reactive immunologic material or CRIM
421 status) of patients requiring factor/enzyme replacement therapies for risk evaluation
422 and mitigation.

423

424 • Evaluation of the extent of polymorphisms, including single nucleotide
425 polymorphisms, in patient populations to identify potential mismatches with the
426 therapeutic protein product.

427

428

429 **B. Product-Specific Factors That Affect Immunogenicity**

430

431 *1. Product Origin (foreign or endogenous)*

432

433 Immune responses to nonhuman (i.e., foreign) proteins are expected, and, as explained above,
434 may be anticipated for some endogenous proteins. Moreover, mismatches between the
435 sequence of the endogenous protein of the patient and that of the therapeutic protein product
436 due to naturally occurring polymorphisms are a risk factor for the development of immune
437 responses to the therapeutic protein product (Viel, et al. 2009). However, the rapidity of
438 development, the strength (titer), and the persistence of the response may depend on a number
439 of factors, including the following: previous and ongoing environmental exposure and the mode
440 of such exposure; the presence in the product of immunity-provoking factors, such as product
441 aggregates and materials with adjuvant activity; and the product's inherent immunomodulatory
442 activity (see section 6 below). For example, environmental exposure to bacterial proteins from
443 either commensal or pathogenic bacteria on skin or in the gut may predispose to generation of

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444 immune responses when such bacterial proteins (either recombinantly or naturally derived) are
445 used as therapeutics.

446
447 For proteins derived from natural sources, antibodies can develop not only to the desired
448 therapeutic protein product, but also to other foreign protein components potentially present in
449 the product. For example, during treatment with a bovine thrombin product, immune responses
450 to bovine coagulation factor V, present in the product, led to development of antibodies that
451 cross-reacted against human-Factor V and resulted in life-threatening bleeding in some patients
452 (Kessler and Ortel 2009). Sponsors investigating such products should thus evaluate the risk
453 posed by immune responses not only to the therapeutic moiety, but also to any known protein
454 or other impurities that may be present.

455
456 *Recommendation*

457
458 Naturally sourced products should be evaluated for other components, protein and non-protein.
459 A risk-based evaluation of immunogenicity of process and product related impurities should be
460 performed and a testing program designed based on such an evaluation.

461
462 2. *Primary Molecular Structure/Post Translational Modifications*

463
464 Both the primary sequence and the higher-order structure of therapeutic protein products are
465 important factors that contribute to immunogenicity. Primary sequence analysis can reveal
466 potentially immunogenic sequence differences in proteins that are otherwise relatively
467 conserved between humans and animals. In such cases, the nonhuman epitopes may elicit T
468 cell help or facilitate epitope spreading to generate an antibody response to the conserved
469 human sequences (Dalum, et al. 1997). Primary sequence analyses may also reveal
470 polymorphisms in relatively conserved human proteins that could lead to immune responses in
471 patients whose endogenous protein amino acid sequence differs from that of the therapeutic
472 protein product.

473
474 More advanced analyses of primary sequence are also likely to detect HLA class II binding
475 epitopes in nonpolymorphic human proteins. Such epitopes may elicit and activate regulatory T
476 cells which enforce self-tolerance, or, opposingly, could activate T helper (Th) cells when
477 immune tolerance to the endogenous protein is not robust (Weber, et al. 2009; Barbosa and
478 Celis 2007; Tatarewicz, et al. 2007; De Groot, et al. 2008). However, engineering of changes to
479 the primary sequence to eliminate immunogenic Th cell epitopes or addition of toleragenic T
480 cell epitopes should be done cautiously, because these modifications may alter critical product
481 quality attributes such as propensity to aggregate, and susceptibility to deamidation and
482 oxidation, and thus alter product stability. Therefore, extensive evaluation and testing of
483 critical product attributes should be performed following such changes. Primary sequence
484 considerations are especially important in evaluation of the immunogenicity of fusion proteins,
485 because immune responses to neoantigens formed from the joining region may be elicited
486 (Miller, et al. 1999) and may then spread to conserved segments of the molecule. Fusion
487 proteins consisting of a foreign protein and an endogenous protein are of particular concern
488 because of the capacity of the foreign protein to elicit T cell help for generation of an antibody
489 response to the endogenous protein partner (Dalum, et al. 1997).

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491 Chemical modifications of therapeutic protein products such as oxidation, deamidation,
492 aldehyde modification, and deimination may elicit immune responses by modification of
493 primary sequence, by causing aggregate formation, or by altering antigen processing and
494 presentation. Importantly, such changes may be well controlled during manufacture and
495 storage, but may occur in vivo in the context of the relatively high pH of the in vivo
496 environment or in inflammatory environments, and cause loss of activity as well as elicitation
497 of immune responses. Evaluation of therapeutic protein products in the context of the in vivo
498 environments to which they are targeted can reveal susceptibility to chemical degradation that
499 may contribute to loss of activity and increased immunogenicity (Demeule, Gurny, et al. 2006;
500 Makrygiannakis, et al. 2006; Huang, et al. 2005). Susceptibility to chemical modifications of
501 therapeutic protein products, and thus the possibility of loss of activity or induction of immune
502 responses in vivo, should prompt consideration of careful protein engineering.

503

Recommendations

504

505
506 Careful consideration should be given to the primary sequences chosen for development of
507 therapeutic proteins in general and especially of therapeutic protein counterparts of endogenous
508 proteins in view of potential polymorphisms in endogenous proteins across human populations.

509

510 For assessment of immune responses to fusion molecules, or to engineered versions of
511 therapeutic protein products, antibody assays should be developed that enable assessment of
512 responses to the intact protein product, as well as to each of the partner proteins separately or to
513 novel regions. Immune responses directed to the intact protein product, but not reactive with
514 either of the separate partner proteins, may be targeting novel epitopes in the fusion region.

515

516 Evaluation of therapeutic protein products in the in vivo milieu in which they function (e.g., in
517 inflammatory environments or at physiologic pH) may reveal susceptibilities to modifications
518 (e.g., aggregation and deamidation) that result in loss of efficacy or induction of immune
519 responses. Such information may facilitate product engineering to withstand undesirable
520 effects. Sponsors should consider this information in early product design and in development
521 of improved products.

522

3. Quaternary Structure: Product Aggregates and Measurement of Aggregates

524

525 Protein aggregates have been recognized as potent elicitors of immune responses to therapeutic
526 protein products for over a half-century (Gamble 1966). Mechanisms by which protein
527 aggregates facilitate immune responses include the following: extensive cross-linking of B cell
528 receptors, causing efficient B cell activation (Dintzis, et al. 1989; Bachmann, et al. 1993);
529 enhancing antigen uptake, processing, and presentation; and triggering immunostimulatory
530 danger signals (Seong and Matzinger 2004), thus recruiting the T cell help needed for
531 generation of high-affinity, isotype-switched IgG antibody, the antibody response most often
532 associated with neutralization of product efficacy (Bachmann and Zinkernagel 1997).

533

534 Protein aggregates are composed either of intact native protein or of degraded or denatured
535 protein which has lost epitopes of the normal protein. Antibodies generated by aggregates

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536 containing native protein can bind to monomeric protein as well, with the potential to inhibit or
537 neutralize product activity. In contrast, antibodies to denatured/degraded protein bind uniquely
538 to the aggregated material, but not to native protein monomers, such as was the case with early
539 preparations of human intravenous immune globulin (IVIG) (Barandun, et al. 1962; Ellis and
540 Henney 1969). Such responses have been shown to cause anaphylaxis, but do not inhibit or
541 neutralize activity of the native protein.

542

543 Critical information is lacking regarding the types and quantities of aggregates needed to
544 generate immune responses for any given therapeutic protein product, although it is generally
545 recognized that higher-molecular-weight aggregates (i.e., >100 kD) and particles are more
546 potent in eliciting such responses than lower-molecular-weight aggregates (Bachmann, et al.
547 1993). The aggregates formed and the quantities that efficiently elicit immune responses also
548 may differ for different products and in different clinical scenarios. Furthermore, the use of any
549 single method for assessment of aggregates is not sufficient to provide a robust measure of
550 protein aggregation. For example, sole use of size exclusion chromatography may preclude
551 detection of higher-molecular-weight aggregates that fail to traverse the column prefilter, yet
552 may be the most crucial species in generating immune responses. Moreover, it has been
553 recognized that subvisible particulates in the size range of 0.1-10 microns have a strong
554 potential to be immunogenic, but are not precisely monitored by currently employed
555 technologies (Berkowitz 2006; Wyatt Technology n.d.; Gross and Zeppezauer 2010; Roda,
556 et al. 2009; Mahler and Jiskoot 2012). These very large aggregates may contain thousands to
557 millions of protein molecules and may be homogeneous or heterogeneous (e.g., protein
558 molecules adhered to glass or metal particles).

559

Recommendations

560

561
562 It is critical for manufacturers of therapeutic protein products to minimize protein aggregation
563 to the extent possible. This can be done by using an appropriate cell substrate, selecting
564 manufacturing conditions that minimize aggregate formation, employing a robust purification
565 scheme that eliminates aggregates, and choosing a formulation and container closure that
566 minimizes aggregation during storage. It is particularly important that product expiration
567 dating take into account any increase in protein aggregates associated with protein denaturation
568 or degradation during storage.

569

570 Methods that individually or in combination enhance detection of protein aggregates should be
571 employed to characterize these distinct species of aggregates in a product. One or more such
572 assays should be validated for use in routine lot release, and several of them should be
573 employed for comparability assessments. Methods include, but are not limited to the following:
574 size exclusion chromatography, analytical ultracentrifugation (Berkowitz 2006), light scattering
575 techniques (Wyatt Technology n.d.), Fourier transformed infrared spectroscopy (Gross and
576 Zeppezauer 2010), and field flow fractionation (Roda, et al. 2009).

577

578 Assessment should be made of the range and levels of subvisible particles (2-10 microns)
579 present in therapeutic protein products initially and over the course of the shelf life. Several
580 methods are qualified to evaluate the content of subvisible particulates in this size range
581 (Mahler and Jiskoot 2012). Sponsors should conduct a risk assessment of the impact of these

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582 particles on the clinical performance of the therapeutic protein product and develop a mitigation
583 strategy based on that assessment, when appropriate.

584

585 4. *Glycosylation/Pegylation*

586

587 Glycosylation may strongly modulate immunogenicity of therapeutic protein products.
588 Although foreign glycoforms such as mammalian xenogeneic sugars (Chung, et al. 2008;
589 Ghaderi, et al. 2010), yeast mannans (Bretthauer and Castellino 1999), or plant sugars (Gomord
590 and Faye 2004) may trigger vigorous innate and acquired immune responses, glycosylation of
591 proteins with conserved mammalian sugars generally enhances product solubility and
592 diminishes product aggregation and immunogenicity. Glycosylation indirectly alters protein
593 immunogenicity by minimizing protein aggregation, as well as by shielding immunogenic
594 protein epitopes from the immune system (Wei, et al. 2003; Cole, et al. 2004). Pegylation of
595 therapeutic protein products has been found to diminish their immunogenicity via similar
596 mechanisms (Inada, et al. 1995; Harris, Martin, et al. 2001), although immune responses to the
597 polyethylene glycol (PEG) itself have been recognized and have caused loss of product efficacy
598 and adverse safety consequences (Lui, et al. 2011). Anti-PEG antibodies have also been found
599 to be cross-reactive between pegylated products.

600

601 *Recommendations*

602

603 For proteins that are normally glycosylated, use of a cell substrate production system that
604 glycosylates the protein in a nonimmunogenic manner and close to the normal human pattern is
605 recommended.

606

607 For pegylated therapeutic proteins, assays for antibodies to PEG itself should be developed and
608 implemented concomitantly with antibody assays to the therapeutic protein.

609

610 5. *Impurities with Adjuvant Activity*

611

612 Adjuvant activity can arise through multiple mechanisms, including the presence of microbial
613 impurities in therapeutic protein products. These innate immune response modulating
614 impurities (IIRMI), including lipopolysaccharide, β -glucan, and flagellin, exert immune
615 enhancing activity by binding to, and signaling through, Toll-like receptors or other pathogen
616 recognition receptors present on B cells, dendritic cells, and other antigen presenting cell
617 populations (Verthelyi and Wang 2010; Iwasaki and Medzhitov 2010). This signaling prompts
618 maturation of antigen presenting cells and/or serves to directly stimulate B cell antibody
619 production. It is very important to minimize the types and amounts of such microbial
620 impurities in therapeutic protein products.

621

622 *Recommendations*

623

624 Assays to evaluate the types of IIRMI present should be tailored to the relevant cell substrate.
625 Because even trace levels of IIRMI can modify the immunogenicity of a therapeutic protein
626 product, the assays used to detect them should have sensitivities that are clinically relevant.

627

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628 Biomarkers used to detect and compare the presence of IIRMI should be tailored to the IIRMI
629 that could be present in the product.

630

631 6. *Immunomodulatory Properties of the Therapeutic Protein Product*

632

633 The immunologic activity of any given therapeutic protein product critically influences not only
634 the immune response directed to it, but also immune responses directed to other co-
635 administered therapeutic protein products, endogenous proteins, or even small drug molecules,
636 and may not be predictable. For example, interferon-alpha (Gogas, et al. 2006; Tovey and
637 Lallemand 2010), interleukin-2 (Franzke, et al. 1999), and GM-CSF (Hamilton 2008) are not
638 only relatively immunogenic of themselves, but also are known to upregulate immune
639 responses to endogenous proteins and to induce clinical autoimmunity. Immunosuppressive
640 therapeutic proteins may globally downregulate immune responses, raising the possibility of
641 serious infections. However, not all immunosuppressive therapeutic proteins suppress
642 responses to themselves. For example, integrin and TNF monoclonal antibodies tend to be
643 immunogenic. Thus, the immunogenicity of such protein therapeutics should be evaluated
644 empirically.

645

646 *Recommendations*

647

648 The immunomodulatory properties of therapeutic protein products, their effects on immune
649 responses to themselves, and their capacity to induce autoimmunity should be monitored from
650 the earliest stages of product development.

651

652 Vaccination using live attenuated organisms should be avoided when the therapeutic protein
653 product is immunosuppressive. Updated vaccination status, compliant with local healthcare
654 standards, is recommended for patients before administration of the therapeutic protein product.

655

656 7. *Formulation*

657

658 Formulation components are principally chosen for their ability to preserve the native
659 conformation of the protein in storage by preventing denaturation due to hydrophobic
660 interactions, as well as chemical degradation, including truncation, oxidation, and deamidation
661 (Cleland, Powell, et al. 1993; Shire, Shahrokh, et al. 2004; Wakankar and Borchardt 2006).
662 Large protein excipients in the formulation, such as human serum albumin (HSA), may affect
663 immunogenicity positively or negatively. Excipients such as HSA, although added for their
664 ability to inhibit hydrophobic interactions, may coaggregate with product or form protein
665 adducts under suboptimal storage conditions (Braun and Alsenz 1997). Polysorbate, a nonionic
666 detergent, is the most commonly used alternative to HSA because its association with proteins
667 minimizes hydrophobic interactions. The stability of both types of excipients (i.e., HSA and
668 polysorbate) should be kept in mind for formulation purposes because they too are subject to
669 modifications (e.g., oxidation), which may then pose a threat to the integrity of the therapeutic
670 protein product.

671

672 Formulation may also affect immunogenicity of the product by causing leaching of materials
673 with immune adjuvant activity from the container closure system. Organic compounds with

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674 immunologic activity as well as metals have been eluted from container closure materials by
675 polysorbate-containing formulations leading to increased oxidation and aggregation.

676
677 *Recommendations*

678
679 Excipients should be evaluated for their potential to prevent denaturation and degradation of
680 therapeutic protein products during storage. Interactions between excipients and therapeutic
681 proteins should be carefully evaluated, especially in terms of co-aggregation or formation of
682 product-excipient adducts.

683
684 Excipient stability should be carefully considered when establishing product shelf life.
685 Thorough analyses of leachables and extractables should be performed to evaluate the capacity
686 of container closure materials to interact with and modify the therapeutic protein product. An
687 appropriate risk mitigation strategy should be developed, as appropriate, following such an
688 assessment.

689
690 8. *Container Closure Considerations*

691
692 Interactions between therapeutic protein products and the container closure may negatively
693 affect product quality and immunogenicity. These interactions are more likely with prefilled
694 syringes of therapeutic protein products. These syringes are composed of multiple surfaces and
695 materials that interact with product over a prolonged time period and thus have the potential to
696 alter product quality. Other container closure considerations that are pertinent to
697 immunogenicity include the following:

- 698
- 699 • Glass and air interfaces are hydrophobic surfaces that can denature proteins and
700 cause aggregation in glass syringes and vials.
701
 - 702 • Glass vials have been known to delaminate at higher pH and with citrate
703 formulations, potentially creating protein-coated glass particles, which may enhance
704 immunogenicity of the therapeutic protein (Frandsen, Carpenter, et al. 2011).
705
 - 706 • Silicone oil-coated syringe plungers provide a chemical and structural environment
707 on which proteins can denature and aggregate.
708
 - 709 • Leached materials from the container closure system may be a source of materials
710 that enhance immunogenicity, either by chemically modifying the therapeutic
711 protein product, or by having direct immune adjuvant activity, including the
712 following:
713
 - 714 ○ Organic compounds with immunomodulatory activity may be eluted from
715 container closure materials by polysorbate-containing formulations: a
716 leachable organic compound involved in vulcanization was found in a
717 polysorbate formulated product when the stopper surfaces were not teflon
718 coated (Boven, et al. 2005).
719

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- 720 ○ Metals that oxidize and aggregate therapeutic protein products or activate
721 metalloproteinases have been found in various products contained in
722 prefilled syringes or in vials. For example, tungsten oxide that leached from
723 the syringe barrel was reported to cause protein aggregation (Bee, et al.
724 2009) and leached metals from vial stoppers caused increased proteolysis of
725 a therapeutic protein due to activation of a metalloprotease that co-purified
726 with the product.

727

Recommendations

728

729
730 Sponsors should obtain a detailed description of all raw materials used in manufacture of the
731 container closure systems for their products. Assays based on such techniques as reverse-phase
732 high-performance liquid chromatography should be developed and used to assess the presence
733 of leachables in therapeutic protein products.

734

735 Because the United States Pharmacopeia “elastomeric closures for injections” tests do not
736 adequately characterize the impact of leachables in storage containers on therapeutic protein
737 products under real-time storage conditions, leachables must be evaluated for each therapeutic
738 protein product in the context of its storage container under real-time storage conditions.

739

740 Testing for leachables should be performed on the product under stress conditions, as well as
741 under real-time storage conditions because in some cases, the amount of leachables increases
742 dramatically over time and at elevated temperatures. Product compatibility testing should be
743 performed to assess the effects of container closure system materials and all leachables on
744 product quality.

745

9. Product Custody

746

747
748 Products formulated in prefilled syringes should be tested for stability in protocols that include
749 appropriate in-use conditions (e.g., light and temperature) to identify conditions and practices
750 that cause product degradation.

751

752 Given that most therapeutic protein products denature and aggregate on exposure to heat and
753 light, or with mechanical agitation, to ensure product quality, patients should be educated
754 regarding product storage, handling, and administration.

755

756 A secure supply chain is critical. Cold chain security is of utmost importance in preserving
757 product quality. For example, the custody of epoetin- α by unauthorized vendors was associated
758 with high levels of aggregates and antibody-mediated pure red cell aplasia (Fotiou, et al. 2009).

759

Recommendations

760

761
762 Patient educational materials (e.g., FDA-approved patient labeling providing instructions for
763 use as required under 21 CFR 201.57 and 201.80) should explicitly identify appropriate storage
764 and handling conditions of the product. Appropriate patient instruction by caregivers is vital to

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765 ensure product quality and help minimize adverse events. Cold chain security should be
766 ensured.

767

768

769 **VI. CONCLUSION**

770

771 Consequences of immune responses to therapeutic proteins can range from no apparent effect to
772 serious adverse events, including life-threatening complications, such as anaphylaxis,
773 neutralization of the effectiveness of life-saving or greatly needed therapies, or neutralization of
774 endogenous proteins with nonredundant functions. Although immunogenicity risk factors
775 pertaining to product quality attributes and patient/protocol factors are understood, immune
776 responses to therapeutic proteins cannot be predicted based solely on characterization of these
777 factors but should be evaluated in the clinic. A risk-based approach, as delineated in this
778 guidance, provides investigators with the tools to develop novel protein therapeutics, evaluate
779 the effect of manufacturing changes, and evaluate the potential need for tolerance-inducing
780 protocols when severe consequences result from immunogenicity.

781

782

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1000 **VIII. APPENDIX**

1001

1002 **A. Diagnosis of Anaphylaxis**

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1004 The diagnosis of anaphylaxis is based on the following three clinical criteria, with anaphylaxis
1005 considered as highly likely when one of these criteria is fulfilled: (Sampson, et al. 2006):

1006

1007 1. Acute onset of an illness (minutes to several hours) with involvement of the skin,
1008 mucosal tissue, or both (e.g., generalized hives, pruritus, or flushing, swollen lips-
1009 tongue-uvula) and at least one of the following

1010 • Respiratory compromise (e.g., dyspnea, wheezing/bronchospasm, stridor, reduced
1011 peak expiratory flow on pulmonary function testing, hypoxemia)

1012 • Reduced blood pressure or associated symptoms of end-organ dysfunction (e.g.,
1013 hypotonia (collapse), syncope, incontinence)

1014

1015 2. Two or more of the following that occur rapidly after exposure to a likely allergen for
1016 that patient (minutes to several hours)

1017 • Involvement of the skin-mucosal tissue (e.g., generalized hives, itching-flushing,
1018 swollen lips-tongue-uvula)

1019 • Respiratory compromise (e.g., dyspnea, wheezing/bronchospasm, stridor, reduced
1020 peak expiratory flow, hypoxemia)

1021 • Reduced blood pressure or associated symptoms (e.g., hypotonia (collapse),
1022 syncope, incontinence)

1023 • Persistent gastrointestinal symptoms (e.g., crampy abdominal pain, vomiting)

1024

1025 3. Reduced blood pressure after exposure to known allergen for that patient (minutes to
1026 several hours)

1027 • Infants and children: low systolic blood pressure (age specific) or greater than 30%
1028 decrease in systolic blood pressure

1029 • Adults: systolic blood pressure of less than 90 mm Hg or greater than 30% decrease
1030 from that person's baseline

1031

1032 Although none of the clinical criteria provide 100% sensitivity and specificity, it is believed
1033 that these criteria are likely to capture more than 95% of cases of anaphylaxis.

1034

1035

1036 Laboratory tests for evaluating anaphylaxis:

1037

1038 At present, there are no sensitive and specific laboratory tests to confirm the clinical diagnosis
1039 of anaphylaxis. Skin testing and in vitro diagnostic tests to determine the level of specific IgE
1040 antibodies directed against the therapeutic protein may be useful for determining whether
1041 anaphylaxis is IgE-mediated. However, the results of unvalidated tests should be interpreted
1042 with caution and the clinical relevance of positive results may be uncertain during product
1043 development. Skin test methods should include positive and negative controls and delineate
1044 criteria for positive vs. negative skin reactions. The input of resources to develop and validate a
1045 prick and/or intradermal skin test for a respective therapeutic protein product (i.e., the

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1046 demonstration of high sensitivity and specificity) should be balanced by the utility of these tests
1047 in the confirmation of the diagnosis of anaphylaxis.

1048
1049 In vitro diagnostic tests that may be employed to determine the level of specific IgE antibodies
1050 directed against the therapeutic protein are the solid-phase radioallergosorbent test (RAST) and
1051 enzymatic assays (Sampson, et al. 2006). As with skin testing, application of such assays for
1052 evaluation of small molecule drugs or peptide therapeutics may be limited due to insufficient
1053 information about relevant metabolites or haptened forms. RAST is of particular use in a
1054 number of situations: extensive skin disease, drug inhibition, and patient fear of skin testing.
1055 The presence of very high levels of nonspecific IgE can yield false positive results, whereas
1056 presence of IgG with the same specificity can yield false negative results via a ‘blocking
1057 antibody’ effect.

1058
1059 Plasma or urine histamine concentrations and total tryptase concentrations in serum or plasma
1060 may help support a clinical diagnosis of anaphylaxis and the pathophysiologic role of mast cell
1061 degranulation. However, these tests have intrinsic limitations (Simons 2009; Simons, et al.
1062 2007; Sanz, et al. 2010). Accurate measurement of these mediators warrants careful timing of
1063 sampling and proper storage of the serum. Appropriate and meaningful interpretation of these
1064 results depends on the clinical context.

1065
1066 In humans, plasma histamine levels are reported to peak 5 to 15 minutes after an IgE-mediated
1067 anaphylactic episode and to return to baseline by 30 to 60 minutes. However, the accuracy of
1068 plasma histamine levels is limited by the requirement for immediate processing to prevent
1069 spontaneous basophil histamine release and the resulting artifactually elevated histamine levels
1070 that occur in unseparated blood. Urinary histamine and its metabolites are elevated for a longer
1071 period following an anaphylactic episode and, therefore, measurements of these levels may
1072 prove useful (Simons, et al. 2007; Lieberman, et al. 2010).

1073
1074 Similarly, tryptase levels may support the role of mast cell degranulation in an anaphylactic
1075 reaction. The majority of constitutively secreted tryptase is β -pro tryptase, an immature β
1076 tryptase, with α -tryptase contributing only a small amount. The marked increase in total
1077 tryptase observed during anaphylaxis is due to the rise in the mature β tryptase on degranulation
1078 (Lieberman, et al. 2010). Currently available tryptase assays detect both α - and β -tryptase, with
1079 a normal level below 11 ng/mL. During anaphylaxis, serum levels of β -tryptase have been
1080 reported to peak 30 to 60 minutes after the onset of symptoms and then decline, with a half-life
1081 of approximately 2 hours. The sensitivity and specificity of the assay may be enhanced if a 2-
1082 fold or greater increase in total tryptase over baseline levels is observed during the acute event.
1083 Baseline serum tryptase levels may be obtained either before the anaphylaxis event in question
1084 or 24 or more hours after resolution of clinical signs and symptoms (Shanmugam, et al. 2006).
1085 It should be noted that although an elevated total tryptase level supports the diagnosis of
1086 anaphylaxis, failure to document an elevation in total tryptase does not exclude the diagnosis
1087 even if the blood sample has been obtained within a few hours of the onset of symptoms
1088 (Simons, et al. 2007). Moreover, tryptase levels are elevated in patients with systemic
1089 mastocytosis. Therefore, mastocytosis should be excluded in the context of elevated tryptase
1090 levels during anaphylaxis (Brockow and Metcalfe 2010). Lack of correlation between

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1091 histamine and tryptase levels in anaphylaxis has been reported, with some patients exhibiting
1092 elevations of only one of these mediators (Sampson, et al. 2006).

1093
1094 Although only 42% of patients given the clinical diagnosis of anaphylaxis were found to have
1095 increased plasma histamine levels, and only 21% had increased plasma tryptase levels (Lin,
1096 et al. 2000), elevated mast cell mediators in the clinical setting of an anaphylactic episode
1097 strongly support the clinical diagnosis, especially if serial sampling demonstrates a significant
1098 change at the time of the inciting event when compared to baseline or post-recovery serum
1099 (Simons 2008). Other tests of immune responsiveness, such as T cell proliferation assays, are
1100 insufficiently specific to serve as indicators or predictors of anaphylaxis.

1101

1102

1103 **B. Cytokine Release Syndrome**

1104

1105 Antibodies to therapeutic protein products have the potential to cross-link membrane-bound
1106 therapeutic proteins such as monoclonal antibodies (mAbs), possibly resulting in augmentation
1107 of a product's intrinsic agonist activity and exacerbation of infusion-related toxicities. In vitro
1108 assessments of cellular activation, including proliferation and cytokine release in human whole
1109 blood or peripheral blood mononuclear cells are recommended. For products with the potential
1110 to incur a cytokine release syndrome (e.g., receptors and products that either stimulate or
1111 demonstrate the ability to induce in vitro or in vivo cytokine release), an initial starting dose
1112 below that obtained by traditional calculations and slower infusion rates, where applicable, may
1113 also be recommended (Duff 2006). Pre- and post-administration levels of C-reactive protein
1114 and cytokines, such as TNF- α , IL-2, IL-6, IL-10 and IFN- γ , may serve as markers of a
1115 proinflammatory response.

1116

1117 Data from animal studies may provide information to guide development of therapeutic protein
1118 products with the potential to induce cytokine release. Although the traditional animal models
1119 used for toxicology testing (i.e., rat, mouse, dog, and cynomolgus monkey) rarely demonstrate
1120 overt toxicities related to lymphocyte activation and cytokine release, specific markers related
1121 to T cell activation and cytokine release can be measured in routine toxicology studies,
1122 provided that the drug is pharmacologically active in the test species. These data may then be
1123 useful for predicting the potential for these agents to induce a cytokine release syndrome in the
1124 clinic, or for evaluating the activity of second-generation agents that have been modified to
1125 reduce their level of T cell activation. For example, cytokine production can be measured in
1126 blood samples obtained from treated animals during pharmacokinetic or general toxicology
1127 studies, provided that the amount of samples obtained does not compromise the health of the
1128 animals or the ability to evaluate the toxicology endpoints at study termination. When
1129 evaluation of cytokine release is included in animal testing, measurement of a cytokine panel
1130 that is as broad as possible and includes IL-6, IFN- γ and TNF- α , as well as other relevant
1131 cytokines indicative of cytokine release syndrome is recommended. Such proposed animal
1132 studies should be discussed with FDA prior to initiation (Hsu, et al. 1999; Norman, et al. 2000).
1133 Data from animal studies should be supplemented by in vitro assessments of cellular activation,
1134 including proliferation and cytokine release in human whole blood or peripheral blood
1135 mononuclear cells (Stebbins, et al. 2007; Hellwig, et al. 2008; Romer, et al. 2011). The
1136 impact of product cross-linking should be considered in such studies. Signs of cellular

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1137 activation in vitro should also be taken as an indication that the product has the potential to
1138 induce toxicities in the clinic, regardless of negative findings from preclinical animal studies.

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1140

C. Non-Acute Immune Responses

1142

1143 Type III hypersensitivity responses, including those mediated by immune complexes and T
1144 cells (delayed hypersensitivity responses in the older literature), are relatively rare with respect
1145 to therapeutic protein products and a high degree of clinical suspicion is necessary for the
1146 diagnosis (Hunley, et al. 2004; Dharnidharka, et al. 1998; Goto, et al. 2009; Gamarra, et al.
1147 2006). Signs and symptoms of immune complex deposition may include fever, rash, arthralgia,
1148 myalgia, hematuria, proteinuria, serositis, central nervous system complications, and hemolytic
1149 anemia. Immune complexes, composed of antibody and a therapeutic protein product have
1150 been responsible for development of glomerulonephritis and nephrotic syndrome in patients
1151 undergoing tolerance induction treatment (with factor IX and α -glucosidase) in the face of a
1152 high titer and sustained antibody response (Hunley, et al. 2004; Dharnidharka, et al. 1998).
1153 There have been case reports of immune complex disease with immune responses to
1154 monoclonal antibodies (Goto, et al. 2009; Gamarra, et al. 2006) and situations in which large
1155 doses of a monoclonal antibody targeting high levels of a circulating multivalent antigen may
1156 increase the likelihood of immune complex deposition.

1157

1158 If patients develop signs or symptoms suggestive of immune complex disease, appropriate
1159 laboratory assessments for circulating immune complexes should be undertaken and the
1160 administration of the therapeutic protein product suspended. In certain situations, development
1161 of tolerance inducing therapies that eliminate the antibody response may be appropriate prior to
1162 further attempts at treatment.

1163

1164

D. Antibody Responses to Therapeutic Proteins

1166

1167 Antibodies to therapeutic proteins are classified as either neutralizing or binding (non-
1168 neutralizing). Neutralizing antibodies bind to distinct functional domains of the therapeutic
1169 protein and preclude their activity. For example, antibodies to therapeutic enzymes may bind to
1170 either the catalytic site, blocking catalysis of substrate, or to the uptake domain, preventing
1171 uptake of the enzyme into the cell. In rare circumstances, neutralizing antibody may act as a
1172 “carrier” and enhance the half-life of the product and prolong its therapeutic effect. As
1173 discussed in section III of this guidance, non-neutralizing antibodies bind to areas of the
1174 therapeutic protein other than specific functional domains and may exhibit a range of effects on
1175 safety and efficacy: enhanced or delayed clearance of the therapeutic protein, which may
1176 necessitate dosing changes; induction of anaphylaxis; diminished efficacy of the product by
1177 causing uptake of the therapeutic protein into FcR-expressing cells rather than the target cells;
1178 and facilitation of epitope spreading, allowing the emergence of neutralizing antibodies.
1179 However, they may have no apparent effect on either safety or efficacy.

1180

1181 The development of neutralizing antibody is expected with administration of nonhuman
1182 proteins and in patients receiving factor/enzyme replacement therapies to whom such

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1183 therapeutic proteins appear as foreign. However, neutralizing antibody to an endogenous
1184 protein does not always arise in situations in which the endogenous factor is defective or absent
1185 by genetic mutation, as in the case of hemophilia A or lysosomal storage diseases. Neutralizing
1186 antibodies can develop in healthy individuals to some normal endogenous proteins because
1187 immune tolerance to some endogenous proteins is not robust and can be broken with sufficient
1188 provocation. For example, healthy volunteers treated with a thrombopoietin (TPO)-type protein
1189 mounted a neutralizing antibody response to the therapeutic, which cross-reactively neutralized
1190 endogenous TPO, inducing a prolonged state of thrombocytopenia in those formerly healthy
1191 individuals (Li, et al. 2001). Thus, treatment with therapeutic counterparts of endogenous
1192 proteins serving a unique function, or endogenous proteins present at low abundance, must be
1193 undertaken very cautiously. Neutralizing antibody to a therapeutic protein can also be
1194 catastrophic when it neutralizes the efficacy of a life-saving therapeutic such as therapeutic
1195 enzymes for lysosomal storage disorders and immune tolerance induction should be considered
1196 in such circumstances (Wang, et al. 2008).

1197

1198 Loss of efficacy of mAbs in patients due to immune responses to the mAb can be highly
1199 problematic and the clinical consequences should not be minimized. Sponsors may consider
1200 development of immune tolerance induction regimens in such patients.

1201

1202 As discussed in section III.B.5 of the guidance, if the endogenous protein is redundant in
1203 biological function (e.g., Type I interferons), neutralization of the therapeutic and endogenous
1204 protein may not appear to produce an obvious clinical syndrome. However, the more subtle
1205 effects of blocking endogenous factors, even though redundant in some functions, may not be
1206 apparent until the system is stressed, as not all biological functions of a factor may be known or
1207 fully characterized. Moreover, the effects of long-term persistence of neutralizing antibody, as
1208 have been observed, for example, in a small percentage of patients with antibodies to IFN- β
1209 (Bellomi, et al. 2003), would not be known from short-term follow-up and should be studied
1210 longer term. Generally, for products given chronically, one year or more of immunogenicity
1211 data should be evaluated. However, longer-term evaluation may be warranted depending on the
1212 frequency and severity of the consequences. In some cases, these studies may be done in the
1213 postmarket setting. Agreement with the Agency should be sought regarding the extent of data
1214 required before and after marketing.

1215

1216 In some circumstances, antibody responses, regardless of apparent clinical effect, should be
1217 serially followed until the levels return to baseline or an alternative approach is discussed with
1218 the Agency. Moreover, for patients in whom a therapeutic protein appears to lose efficacy, it is
1219 important that an assessment be undertaken to determine whether the loss of efficacy is
1220 antibody mediated.

1221

1222 For patients who develop neutralizing antibodies or are considered at very high risk of
1223 developing neutralizing antibodies to a life saving therapeutic protein (e.g., CRIM negative
1224 patients with a deletion mutation for a critical enzyme who are given enzyme replacement
1225 therapy), consideration should be given to tolerance induction regimens in a prophylactic
1226 setting, before or concomitant with the onset of treatment (Messinger, et al. 2012; Wang, et al.
1227 2008; Mendelsohn, et al. 2009). Given the degree of immune suppression of such regimens,

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1228 although far less than that of a therapeutic regimen to reverse an ongoing response, careful
1229 safety monitoring should be undertaken throughout the duration of the protocol.

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E. Utility Of Animal Studies

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1234 Immunogenicity assessments in animals are conducted to assist in the interpretation of animal
1235 study results and in the design of subsequent clinical and non-clinical studies (for additional
1236 information, see the Guidance to Industry ICH S6(R1): *Preclinical Safety Evaluation of*
1237 *Biotechnology-Derived Pharmaceuticals*,
1238 [http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S6_R1/Step4/
1239 S6_R1_Guideline.pdf](http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S6_R1/Step4/S6_R1_Guideline.pdf)). They are generally limited in their ability to predict the *incidence* of
1240 human immune responses to a therapeutic protein, but they may be useful in describing the
1241 *consequences* of antibody responses, particularly when an evolutionarily conserved,
1242 nonredundant endogenous protein is inhibited by cross-reactive antibodies generated to its
1243 therapeutic protein product counterpart. When available, animal models, including
1244 hyperimmunized mice or gene knock out (KO) mice, can be used to address potential
1245 consequences of inhibition of endogenous proteins. A special case is that of endogenous
1246 proteins that are vital to embryonic or fetal development whose elimination is embryonically
1247 lethal. In such situations, the use of conditional knock out mice may be useful for assessing
1248 potential consequences of neutralizing antibodies. As in human studies, consideration should be
1249 given to the potential transmission of antibodies to developing neonates by breast milk,

1250

1251 In contrast to proteins that mediate biologically unique functions, animal models are generally
1252 not useful for predicting consequences of immune responses to redundant therapeutic protein
1253 products. Mice that are transgenic for genes encoding human proteins, humanized mice (i.e.,
1254 immune-deficient mice with human immune systems), and mouse models of human diseases
1255 are increasingly being developed and may be considered for use to address multiple clinical
1256 issues, including immunogenicity.

1257
1258

F. Comparative Immunogenicity Studies

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1261 The need for, extent, and timing of clinical immunogenicity studies in the context of
1262 evaluating the effects of a manufacturing change will depend on such factors as the extent of
1263 analytical comparability between the product before and after the manufacturing change,
1264 findings from informative comparative animal studies, and the incidence and clinical
1265 consequences of immune responses to the product prior to the manufacturing change. For
1266 example, if the clinical consequence of an immune response is severe (e.g., when the product
1267 is a therapeutic counterpart of an endogenous protein with a critical, nonredundant biological
1268 function or is known to provoke anaphylaxis), more extensive immunogenicity assessments
1269 will likely be needed.

1270

1271 Guidance on development programs for biosimilar products is available in a separate draft
1272 guidance (*Guidance for Industry on Scientific Considerations in Demonstrating Biosimilarity*
1273 *to a Reference Product*, February 2012).

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1274

1275 Guidance on appropriate assay development for immunogenicity testing is available in a
1276 separate draft guidance (*Assay Development for Immunogenicity Testing of Therapeutic*
1277 *Proteins*), in ICH guidance (ICH Q2A,B), and in recent publications (Koren, et al. 2008).
1278