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Bioanalysis

Immunogenicity of therapeutic protein products: current considerations for anti-drug antibody assay in Japan

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Immunogenicity assessment is an important issue for ensuring the safety and efficacy of therapeutic protein products. Although the reliability of the anti-drug antibody (ADA) assay is one of the key points, there are some difficulties in assessing its validity because the analytes are polyclonal antibodies with variable and unknown characteristics. To elucidate the points to consider for the ADA assay, a Japanese research group was established that discusses the issues raised on the immunogenicity assessment. In this review, we first introduce the current situation regarding the development and immunogenicity assessment of therapeutic protein products in Japan. We then present our current view and recommendations on the ADA assay by considering its unique features.

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Therapeutic proteins such as monoclonal antibodies are currently essential in the treatment of cancer, autoimmune disease and other diseases. Many therapeutic protein products intended to address unmet medical needs are being developed worldwide, including in Japan, and therefore, ensuring their safety and efficacy is of paramount importance. Since protein has its intrinsic feature of immunogenicity owing to its structure containing potential B-cell and T-cell epitopes, therapeutic proteins have the potential to induce ADA even if the protein has the same amino acid sequence as endogenous human proteins. Proteins can be recognized as antigens by B cells and are also incorporated and digested by antigen-presenting cells. This process leads to the production of T-cell epitope peptides that are presented on major histocompatibility (MHC) II molecules. Various patient- and product-specific factors are suggested to affect the process of ADA induction [1]. The emergence of ADA in patients can potentially lead to loss of efficacy and/or adverse events. Therefore, immunogenicity risk assessment and risk-mitigating strategies are required during the development of therapeutic protein products [2].

The appropriateness of the ADA assay is a key issue in immunogenicity assessment. The ligand-binding assay (LBA) is often used for detecting ADA in biological samples. Unlike the assay used for drug concentration analysis, the ADA assay has unique features, for example: the actual characteristics of the analyte (human ADA) are variable and unknown; there is no real reference standard: the experimentally prepared positive control for ADA is used as a surrogate reference standard to evaluate and control the assay performance; and the evaluated validation parameters such as sensitivity and drug tolerance limit (DTL) vary depending on the characteristics, mainly the affinity of the used positive control. In addition, matrix components that interfere with the assay in the study samples may vary depending on the disease and/or the individual patients. Therefore, it is practically impossible to completely assess the validity of the ADA assay, and the assay is associated with certain risks of obtaining an inappropriate result. As mentioned in the European Medicines Agency (EMA) guideline [3] and the US FDA draft guidance [4], a tiered approach is commonly used for immunogenicity assessment by considering these risks.

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Guideline for bioanalytical method validation pertaining to LBA was released by the Ministry of Health, Labor and Welfare of Japan [5,6]. However, bioanalysis for ADA is out of its scope and currently, there are no regulatory guidelines on immunogenicity assessment in Japan. Industries are seeking an appropriate strategy for assessing the immunogenicity of the intended products independently by referring to the EMA guideline, FDA draft guidance and certain white papers [7,8]. The immunogenicity research group was established with the support of AMED (Japan Agency for Medical Research and Development) to promote efficient development of therapeutic protein products by elucidating the fundamental issues of immunogenicity assessment and discussing the issues to be considered in ADA assay development, validation and study sample analysis. In this review, we introduce the current situation regarding the development and immunogenicity assessment of therapeutic protein products in Japan and present our views and recommendations for ADA assays.

Overview of the current situation on immunogenicity assessment of therapeutic protein products in Japan

Research & development of therapeutic protein products in Japan

Similar to other countries, more than 130 therapeutic protein products including insulins, somatropins, erythropoietins, enzymes and monoclonal antibodies have been approved in Japan. Some of them, such as tocilizumab, mogamulizumab and nivolumab, were first developed in Japan. In addition, biosimilar products for somatropin, epoetin alfa, filgrastim, insulin glargine, infliximab and rituximab have been approved.

The development of next-generation medicines is of considerable interest for both the government and industries in Japan. Recently, the Japanese government has encouraged the development of innovative medicines, and the funding agency AMED was established in 2015. Our research group intended to elucidate the fundamental issues of immunogenicity assessment are supported by this framework.

Immunogenicity assessment of therapeutic protein products approved in Japan

Bioanalytical methods that were used for the ADA assay of therapeutic protein products approved in Japan are shown in Figure 1. The most popular method for screening and confirmatory assays is the enzyme-linked immunosorbent assay because of its robustness, sensitivity and compatibility with readily available reagents and instruments (Figure 1A). The second is the electrochemiluminescence (ECL) immune assay, which is currently gaining popularity owing to its higher tolerance to co-existing drugs and a large dynamic range. In fact, ECL was used for six monoclonal antibody (mAb) products among seven mAbs approved in 2016, and almost all latest approved mAb products utilized ECL as the ADA assay. Radioimmunoassay and radioimmunoprecipitation assay are used for peptides and hormones. Surface plasmon resonance was used for ADA assays of peptides, cytokines and mAbs. As the neutralizing antibody assay, functional assays, in which neutralization of the biological activity of a drug is directly measured, are mostly used (Figure 1B). The use of competitive LBA as neutralizing antibody assay is increasing for mAb products.

With respect to the study population of clinical trials, immunogenicity is evaluated in both Japanese and populations of the foreign regions. The association between ADA production and HLA (human leukocyte antigen) types was reported with respect to certain therapeutic protein products [9–11]. Since a case of high frequency of ADA production was reported in Japanese, further clarification on the role of unique ethnic factors in ADA generation is required.

Current view & recommendations for immunogenicity assessment

The tiered approach for immunogenicity assessment consisting of screening assay, confirmatory assay and characterization is well accepted in Japan. At this point, the overall strategy for immunogenicity assessment has already been practically harmonized with those of other regions. However, as mentioned above, ADA assays have specific features that are different from those of drug concentration analysis; it was expected to share the concerns among industry researchers and regulators in order to establish a standard concept of immunogenicity assessment. In the research group, the outline of ADA assay methods used in each institute was shared, and issues to be clarified to elucidate the points to consider in immunogenicity assessment were discussed. In the following sections, our current views, recommendations and issues necessary for further clarifications related to ADA assays are shown.

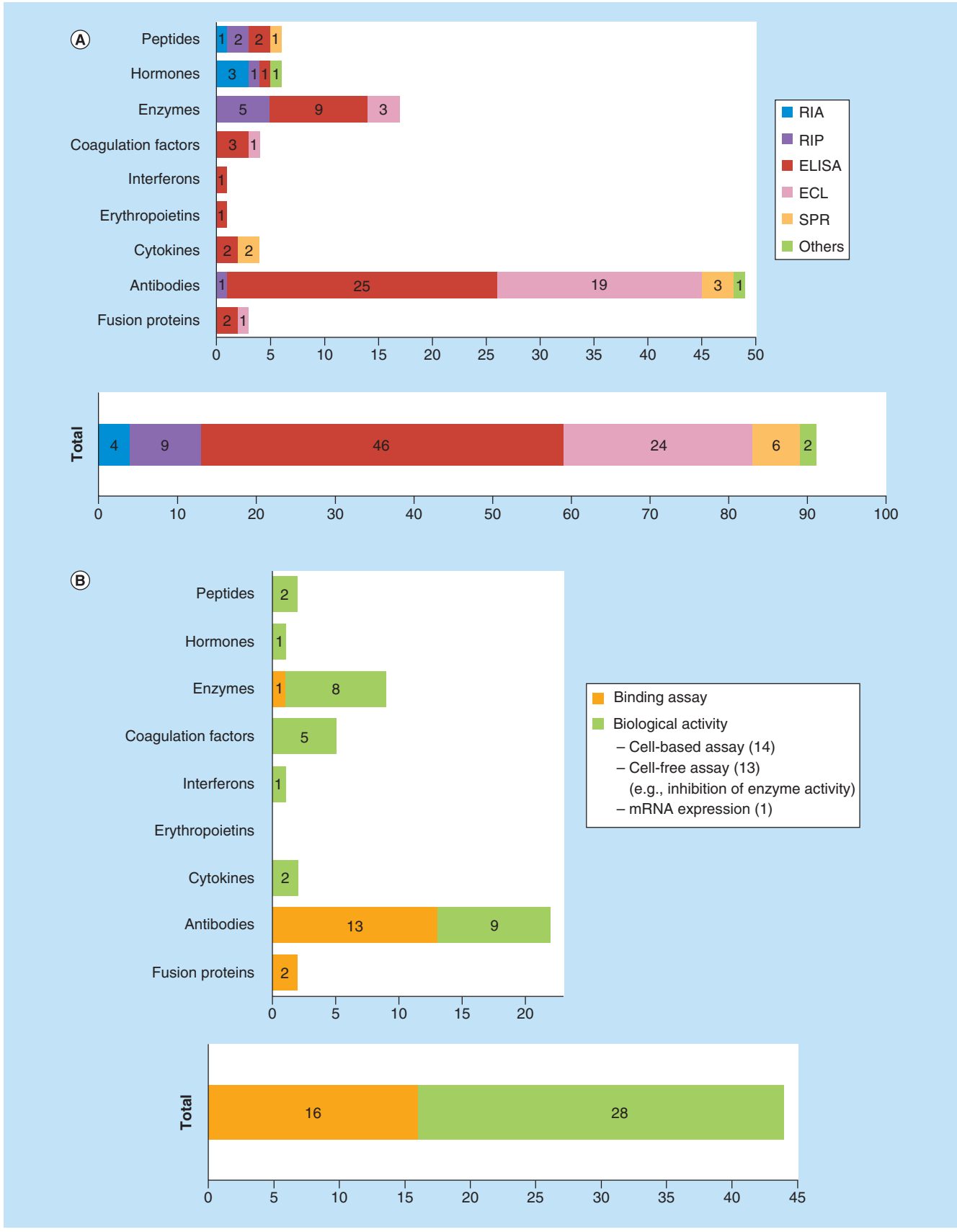

Figure 1. Bioanalytical methods used for the anti-drug antibody assay in immunogenicity assessment of therapeutic protein products approved in Japan. Information was obtained from the review reports or the application dossiers for marketing authorizations disclosed on the website of the Pharmaceuticals and Medical Devices Agency. The data do not cover all products due to the limitation of the disclosed data. **(A)** Screening and confirmatory assays; **(B)** Neutralizing antibody assays.

Positive control

Affinity purified polyclonal or monoclonal antibodies are used as positive controls for method development and validation of ADA assays. It should be noted that the characteristics of the positive control affect the sensitivity and specificity of the ADA assay.

Validation

The fundamental parameters for a bioanalytical method validation of ADA assays are specificity, selectivity, precision, sensitivity, dilution linearity and drug tolerance limit. In addition, the stability of the positive control in spiked samples is usually evaluated to show the consistency of the assay, although it does not guarantee the stability of the ADA in the study samples.

Specificity

The assays should detect only the ADA. Specificity refers to the ability of a method to detect ADA that binds to the drug but not to the assay components such as the assay apparatus (e.g., plates) or the reagents. The specificity can be addressed by demonstrating that binding can be blocked by an unlabeled drug.

Selectivity

The assay results may be affected by interference from the matrix or an existing drug. Selectivity refers to the ability of a method to identify drug-specific ADA in a matrix such as serum or plasma that may contain potential interfering substances (e.g., process-related impurities, human endogenous proteins, substances from erythrocytes and lipids). It should be mentioned that as one of the human endogenous proteins, target molecules of the drug such as the target antigen of a mAb product can cause selectivity problems, resulting in false-positive results (i.e., target interference). The selectivity issue can be addressed by measuring the responses of individual drug-naive negative samples. The use of hemolysis/lipemia samples are also to be considered.

Precision

Assay results should be equivalent within and between assay runs. Precision refers to the ability of a method to measure the variability in a series of measurements for the same material run in a method. To provide reliable estimations, both intra-assay (repeatability) and inter-assay (intermediate precision) variability of assay responses are evaluated.

Sensitivity

The assays should detect ADA at a maximum level which causes no observed clinical event. In the revised FDA draft guidance, the minimum requirement for the sensitivity is recommended to be 100 ng eq/ml [4]. Assay sensitivity represents the lowest concentration at which the ADA preparation consistently produces either a positive result or readout equal to the cutpoint determined for that particular assay. Sensitivity depends on the binding affinity of the positive control. It should be noted that the value of sensitivity depends on the positive control used, i.e. the apparent sensitivity is high when a positive control with high-binding affinity to the drug is used. In other words, the recommended value of the sensitivity is also a relative value.

Dilution linearity

High concentration of ADA may cause a reduction of the signal (i.e., prozone effect, also called hook effect). Dilution linearity refers to the ability of a method to maintain a positive correlation between the ADA concentration and the signal. If the prozone effect was observed by assessing dilution linearity, it is recommended to estimate the upper limit of ADA concentration in which response above the cutpoint can be obtained. In such a case, the study sample should be analyzed using the appropriate dilution ratios in order to avoid false-negative results.

Drug tolerance limit

Since the ADA assays are generally designed to detect uncomplexed ADA, the presence of target drug affects the assay result. Drug tolerance refers to the ability of a method to detect ADA without interference from a co-existing drug. Drug tolerance can be assessed by examining the changes in response when known amounts of positive control ADA were deliberately added to individual ADA-negative control samples in the absence or presence of different quantities of the drug (see the 'Drug tolerance' section for the details).

Cut-point determination for ADA assay

In ADA assays using the tiered approach, a sample whose response in the screening assay is at and above the cut point is defined to be potentially positive. Subsequently, in the confirmatory assay, the specific response of the potentially positive sample to the drug is evaluated based on the degree of inhibition of the response by adding an excess amount of the drug. Therefore, the appropriate setting of the cut points is critical to ensure the reliability of the assay results. Cut-point determination (in many cases the determination of the normalization factor) is usually performed as a part of the validation.

In the research group, all members referred to the white paper [7], the concept in which was consistent with the FDA draft guidance [12], as a method of cut-point setting. Although we are considering the usefulness of other approach using a one-sided lower confidence interval for the assigned percentile value as described in the revised FDA draft guidance [4], the following are the current standard practices in Japan.

Screening assay cut point

The screening cut point is usually set to yield 5% false-positive rate. In most common methods, samples from ≥50 individual drug-naive human subjects (healthy or diseased) are analyzed in more than three runs by the validated ADA assay, followed by calculation of the 95th percentile value. If the data distribution is assessed as normal, the screening cut point of the 95th percentile is calculated as mean + 1.645 × SD (parametric approach). In contrast, if the data distribution is non-normal, normalization of the data by an appropriate data transformation (e.g., logarithmic transformation, taking ratio of signal to negative control [S/N]) and use of parametric approach are recommended.

Next, means and variances are compared among three runs to assess the suitability of fixed, floating or dynamic cut points (Figure 2). Taking the characteristics of LBA into account, the floating cut point that uses the response of the negative control in each run as the reference value is considered most appropriate.

In terms of the outlier exclusion, various approaches (e.g., excluding values higher than × 1.5 or 3 interquartile range by box plot) would be acceptable if scientifically justified and predefined in the standard operating procedure. The approach that all data are included in the cut-point calculation without excluding outliers is also acceptable if it is justified. However, it should be considered that the assigned cut point often becomes higher with outliers included in the calculation, which may increase the risk of obtaining false negative results.

Confirmatory assay cut point

The confirmatory cut point is typically set to yield 1% false-positive rate as recommended in the FDA draft guidance [4] or 0.1% as exemplified in the white paper [7]. In most common methods, samples from ≥50 individual drug-naive human subjects (healthy or diseased) are spiked with an excess amount of drug and analyzed in the same plate as the unspiked counterpart and the % inhibition value for each sample (100 × [1 − spiked/unspiked]) is calculated. When the confirmatory cut point is set as a false positive rate of 1%, it is calculated as 99th percentile (i.e., mean + 2.33 × SD, if the data distribution is assessed as normal) (Figure 3).

In cases where % inhibition values are negative and cannot be subjected to logarithmic transformation, the analysis of spiked/unspiked ratio instead of % inhibition is recommended. The confirmatory cut point is to be set as the mean of the cut point of three assay runs, because the response of the negative control in each run cannot be used as the reference value.

The FDA draft guidance recommends validation of the confirmatory assay in a manner similar to that of the screening assay [4].

Cut point for the target subject samples

The background response in the ADA assay can be different between healthy and disease subjects. It is also possible that the responses differ among target diseases (e.g., the types of tumor). Therefore, it should be pointed out that using the samples from the target disease subjects should be considered for cut-point setting. However, samples from subjects with target disease are not always available, especially in the early development phase. Therefore, we propose a practical approach for cut-point determination using samples from diseased subjects (Figure 4).

In cases where samples from subjects with target disease are available, the background response is compared with those of healthy subjects. If these background responses differ, the cut point should be determined using the target disease subjects. When samples from the target disease subjects are not available, or the positive rate of the

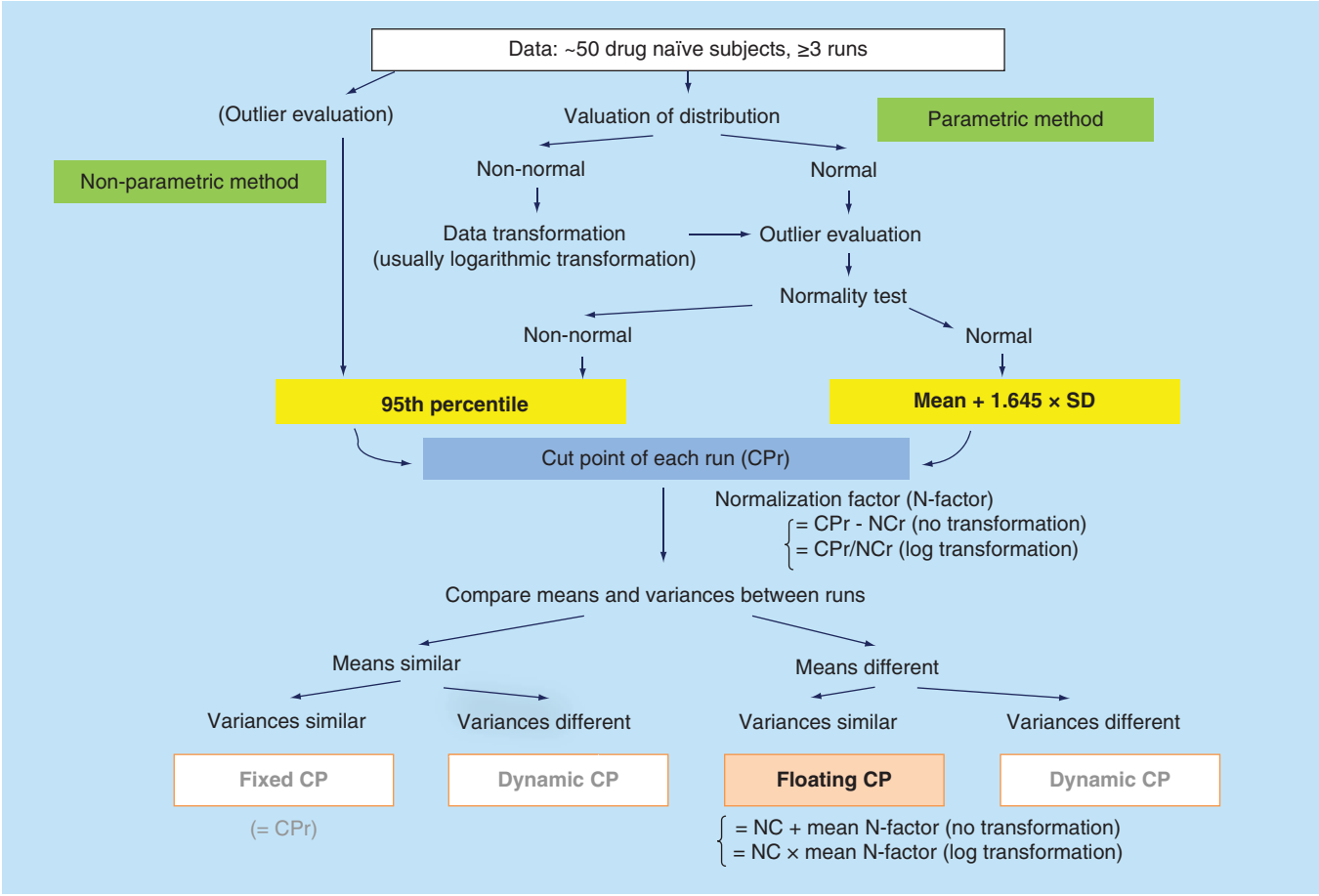

Figure 2. Scheme for setting the screening assay cut point.
NCr: Response of negative controls.
Adapted with permission from [7] © Elsevier (2008).

in-study samples is too high/low, it is recommended to re-evaluate the cut point using predose samples from the study subjects.

Drug tolerance

Drugs that remain in patient samples can form an immune-complex with the generated ADA, which can reduce the assay response and sensitivity [13–15]. This issue is generally more important when the therapeutic protein is an antibody that has a long elimination half-life and high steady-state concentration. The influence of residual drugs on the ADA assay thus needs to be evaluated prior to sample analysis, and methods that overcome this issue should be established as necessary. Several approaches, for example, acid treatment for dissociating immune-complexes and solid-phase extraction for removing excess residual drug in the sample, have already been reported to improve the drug tolerance of ADA assays [16–18]. Long-time incubation and sufficient sample dilution are also effective in reducing the interference [19]. When these approaches are employed, bioanalytical method validation must be conducted including the pretreatment procedure.

The DTL, i.e. the maximum drug concentration at which the detection of ADA is not interfered by the drug, should be determined in the assay validation to assess if the assay is suitable for study sample analysis. We recommend that DTL should exceed at least the anticipated trough drug concentration in clinical use. However, it should be noted that DTL is the apparent concentration obtained with the positive control. The interference in the patient samples may be different from the validation results depending on the characteristics of ADA generated in the patients. In some cases, appropriate sampling schedule for ADA assays, such as sampling during drug holidays, may allow ADA detection even if the DTL of the assay is not sufficient.

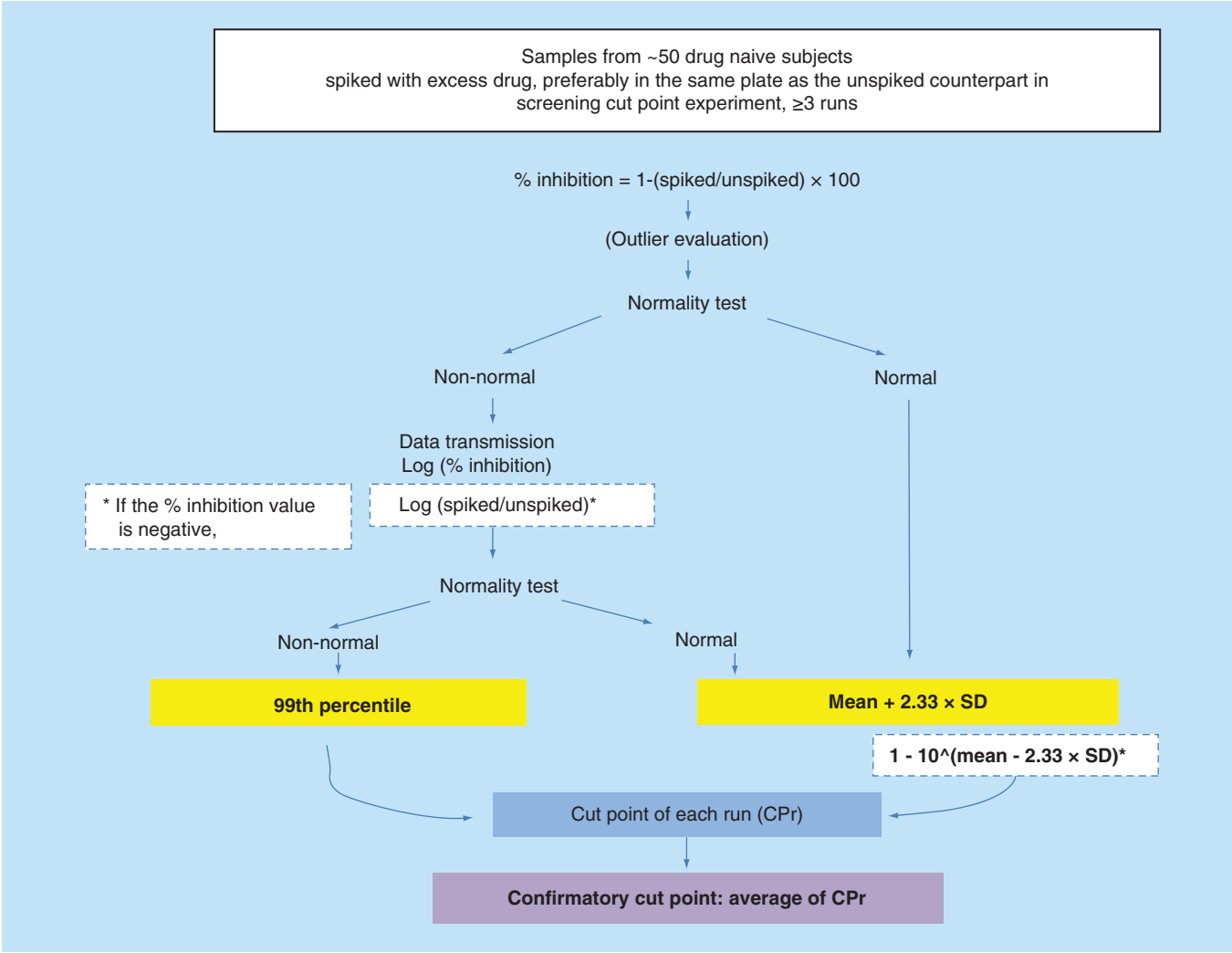

Figure 3. Scheme for setting the confirmatory assay cutpoint.

Despite acid dissociation, solid-phase extraction with acid dissociation and other techniques can improve the DTL dramatically, such pretreatment steps sometimes decrease the assay reproducibility. In addition, such treatments may cause denaturation of ADA in the patient sample, resulting in underestimation of ADA production. The necessity of a pretreatment step for improving DTL should be decided taking the anticipated drug concentration in the patient sample and the advantages/disadvantages of each technique into consideration.

Sampling in clinical trials

We considered that blood sampling should be performed in all pivotal clinical studies. For ADA assays, sampling schedules in the clinical trials are needed to be considered carefully. First, collection of baseline samples before starting drug treatment is recommended to understand the predose levels of ADA (i.e., pre-existing antibodies). Second, sampling schedules would be determined on a case-by-case basis (depending on the pharmacokinetic properties of the drug, such as $T_{1/2}$, administration interval and administration period). Sampling at the trough points of the drug is recommended due to low drug concentrations, which enables low interference to ADA assay, as described in the previous section. In this context, the drug concentration is supposed to be measured at the same time, especially for the low DTL cases. In addition, enough sampling period would be taken to understand whether the detected ADA is present transiently or persistently. It is recommended to take samples for a certain period of time (typically, five half-lives, depending on the drug administration schedules) even after the administration is complete. Third, if ADA or related immunological symptoms are detected, more frequent sampling would be

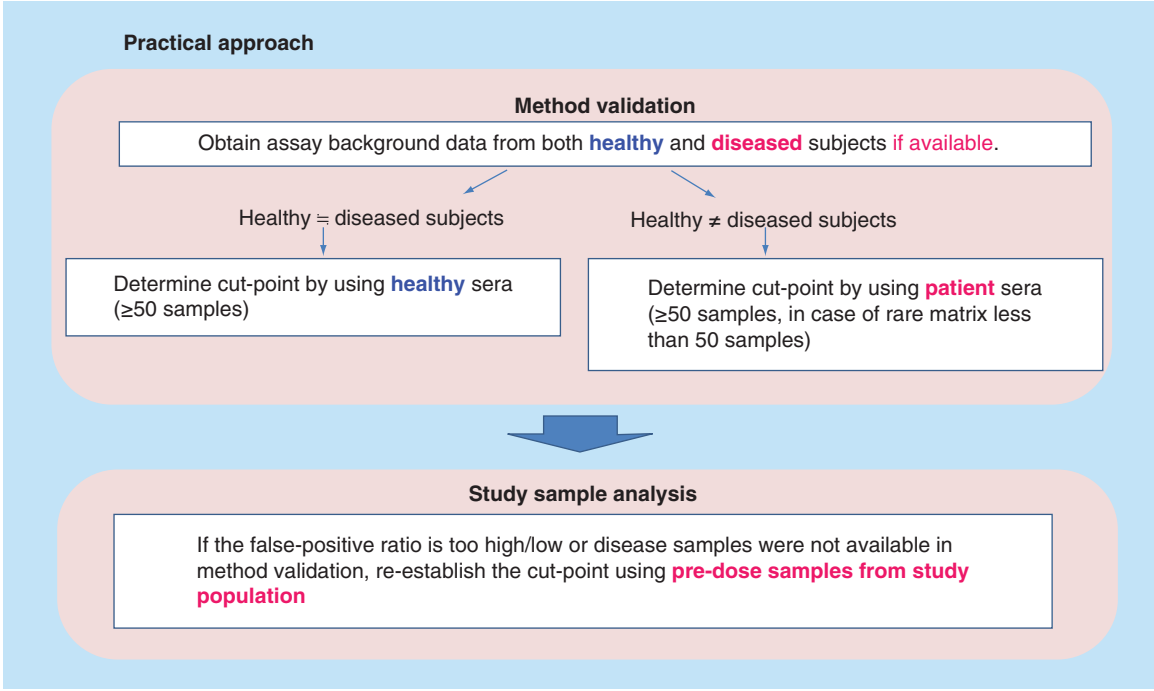

Figure 4. Scheme for setting the cut point focusing on drug-naive samples.

needed to precisely evaluate the impact of the ADA presence, for example, association of ADA levels with the strength of the symptoms. In such cases, the characterization of ADA, including neutralizing properties is also preferred.

As points to consider, we discussed two issues. First, when ADA is detected, information about the isotype and the epitope of ADA may be useful to understand the assay results. Especially, determination of epitope specificity is recommended for nonendogenous fusion molecules, such as antibody–drug conjugates. Second, for multi-regional clinical trials, the frequency of ADA formation could differ among ethnicities as some reports showed an association of highly variable human leukocyte antigen types with ADA formation [20].

The data about ADA and drug concentrations would be described in the application dossiers for marketing authorizations as ADA positive or ADA negative. In addition, we considered that results should be reported as ADA-inconclusive when the concomitant drug concentration is higher than the DTL (even though the assay was optimized to improve the drug tolerance) to avoid missing false-negative results. We recommend reporting whether ADA is positive even before (baseline ADA-positive) or only after treatment (treatment-induced ADA) of the drug, and also whether the ADA level is observed transiently or persistently when the ADA assay results is positive. In the baseline ADA-positive case, it is informative to describe whether the treatment boosts the ADA levels, using a (at least semi-) quantitative assay system.

Immunogenicity assessment in nonclinical study

Many therapeutic proteins administered to humans are exogenous (foreign) for animals and may elicit immune reactions by generating ADA. Although immunogenicity in nonclinical settings may not be predictive for the clinical outcome [21], it should nonetheless be evaluated when altered pharmacodynamical activity, unexpected changes in exposure of drugs or immune-mediated reactions (e.g., immune complex disease, vasculitis and anaphylaxis) are observed as recommended by the International Conference on harmonization S6 (R1) guideline [22]. The measurement of ADA may be helpful for understanding these findings of nonclinical studies. In addition, it is useful to further clarify the ADA characteristics including frequency, titer and neutralizing activity, to interpret pharmacokinetics and toxicity in repeat-dose toxicity studies.

Validation parameters to be assessed for nonclinical ADA assays can be limited compared with those required in clinical settings as they do not directly have an impact on clinical efficacy and safety. Although screening and confirmatory assays are necessary, the evaluation of neutralizing activity and ADA titer may be less important. It

is recommended to evaluate drug tolerance to interpret false-negative ADA results even in nonclinical settings. An alternative nontiered assay, in which the cut point in a screening assay is set at the 99.9th percentile of the standard normal distribution, is proposed to abbreviate a confirmatory assay in immunogenicity assessment [23,24]. Collectively, fit-for-purpose approaches are recommended for nonclinical ADA assays.

Biosimilars

Immunogenicity assessment is also important for the development of biosimilars. The biosimilarity assessment includes evaluation of similarity in quality attributes and clinical aspects. A significant amount of knowledge regarding the quality attributes related to immunogenicity has been accumulated; however, immunogenicity assessment in clinical trials is still deemed as necessary for biosimilars.

In the case of relative immunogenicity assessment, selection of the so-called 'one assay' or 'two assays' is an issue as both methods have their own pros and cons. By using one assay, in which the assay is developed using biosimilar as the labeled reagents, the sensitivity of the assay used for both products is identical, however, ADA binding solely to the innovator cannot be detected using this method. If two assays are used, the ADA for the innovator product and the biosimilar product can be detected in each assay. However, the analytical performance, including sensitivity, may be different between the assays which may make data comparison difficult. Our recommendation is that if the data comparing the innovator and the biosimilar show high similarity in quality attributes, especially for post-translational modifications and higher order structure, the one assay is a suitable approach. In this case, if impurities such as host cell proteins (HCP) have adjuvant activities that make a difference in the immunogenicity between the two products, the difference can be detected even if one assay is used. The anti-HCP antibody is another issue to be considered depending on the host cell used, which requires the development of an anti-HCP antibody assay.

Conclusion & future perspective in Japan

In this review, we summarized the current view and recommendations on the ADA assay discussed in our Japanese research group. We would like to emphasize that the assay performance (showed by validation results) depends on the characteristics of the positive control. In addition, ADA results usually depend on the assay method used. Some methods such as, enzyme-linked immunosorbent assay with several washing steps can preferentially detect high affinity ADA, whereas other methods (e.g., surface plasmon resonance) can detect even low affinity ADA. We should comprehend the ADA assay results in combination with the analytical performance of the assay method.

There is no regulatory guideline for ADA assays in Japan. Many pharmaceutical and contract research organizations are currently developing and performing ADA assays according to FDA draft guidance and EMA guideline. Therefore, establishment of a domestic guideline would be necessary for accelerating the development of therapeutic protein products via smooth evaluation of ADA in Japan, which may lead to the establishment of internationally harmonized guidelines in the future. Standardization of commonly used assay methods, as well as the preparation of publicly available ADA reference panels, may also be anticipated. We hope that this review will help to facilitate the discussion between regulators and industry members in Japan.

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Executive summary

Background

- The anti-drug antibody (ADA) assay has a unique feature that is different from drug concentration analysis. This is because the characteristics of the analytes are variable and there is no reference standard identical to the ADA in the study samples.
- Critical issues for ensuring the reliability of the ADA assay were discussed by a Japanese research group.

Current view & recommendations for ADA assay

- A tiered approach for the ADA assay consisting of screening and confirmatory assays and characterization is well accepted in Japan.
- The fundamental parameters for the bioanalytical method validation of the ADA assays are specificity, selectivity, precision, sensitivity, dilution linearity and drug tolerance.
- Influence of the residual drug in the study sample on detecting ADA should be evaluated, and a strategy for avoiding false-negative results is to be considered.

Conclusion

- It is important to validate the ADA assay appropriately and comprehend its results in combination with the analytical performance of the assay method employed.

Future perspective

- Domestic guideline for immunogenicity assessment would be necessary to promote the development of therapeutic protein products through smooth evaluation of ADA in Japan, which will also be a basis for international harmonization in the future.

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