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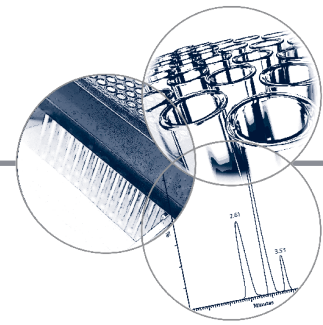

Challenges of immunogenicity assays for vaccines

Clinical development of vaccines requires a specific set of specialized assays to demonstrate the immunogenicity of the vaccine. Ideally, these assays should measure immune responses that correlate with protection against disease. Antibody responses usually correlates to protection for existing vaccines, but for vaccines currently in development it is not always clear which immune responses confer protection. Developing assays for new-generation vaccines usually requires working with cells, pathogens, antigens or assay controls that are not readily available, or are hazardous materials. Validation of these assays involves many challenges, and validation requirements are not yet fully specified in regulatory guidelines or White Papers. The different requirements for clinical vaccine assays and the related challenges in developing and validating these assays are described in this article. We provide our opinion on how to approach these challenges and how to apply the existing guidelines.

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Immunogenicity assays for vaccines are the basis for discovery, research and development of vaccines against infectious diseases. Preclinical research and clinical evaluation of vaccine candidates is usually performed using immunogenicity read-out assays to determine the antibody and T-cell responses. Although in clinical development of such vaccines the preferable clinical end point is protection against infection, reduced morbidity or mortality, these end points can only be adequately assessed in Phase III trials. Since critical decisions need to be made on candidate selection, dose and vaccination schedule before that, such decisions are usually taken based on immunogenicity assessments, which will be the main scope of this article.

Immunogenicity assays for vaccines resemble potency release tests in that they assess the biologically relevant response to the product on a quantitative scale, although they are not intended to release the drug product. They are designed to measure the wanted product's induced immune response in humans, unlike unwanted immunogenicity tests for biologics. Vaccine assays could be categorized as biomarker assays, since they characterize a process that can be measured in the body, or its products, and predict the incidence of outcome or disease [1], that is, 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention' [2]. However, the category of biomarkers is so diverse that we consider vaccine immunogenicity assays as a distinct category within biomarkers that warrants a dedicated approach. The various challenges that are involved in developing, validating and utilizing this category of assays for sample analysis are discussed and we provide our opinion on how to approach these challenges.

Selecting the right assay: correlate of protection

Immunogenicity assays for vaccines can be categorized as a subgroup of biomarker assays. Following the definition of the biomarkers definition working group [2], we can specify vaccine assays as tests that are used for prediction and monitoring of clinical responses to vaccination. The ultimate goal is to predict whether a vaccinee will be protected against infection or disease caused by the pathogen that is targeted by the vaccine. For most existing vaccines, the **correlate of protection** is known to be the antibody response [3]. Although other immune parameters are likely to play a role, the measurement of antibody titers or concentrations is generally accepted as a correlate of protection for diseases such as influenza [4] and hepatitis B [5]. Seasonal influenza vaccines are registered each year based on their ability to induce sufficient hemagglutination, inhibiting antibody titers in serum, without the need for field trials or challenge studies. However, for most vaccines currently in development, the correlate of protection is as yet unknown. For pathogens such as the Malaria parasite, an antibody assay is not sufficient to predict protection and no fully effective vaccine has been developed to date. Identifying the correlate of protection for these

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Key Terms

Immunogenicity: The capacity of a biological substance to induce any form of response by the immune system.

Correlate of protection: A specific immune response to a vaccine that is closely related to protection against infection, disease or other defined end point.

vaccines will increase speed of development of an effective vaccine. Research by investigators all over the world into several immune parameters, such as binding antibody titers, functional antipathogen assays, T-cell Elispot and T-cell culture assays, has revealed much valuable information. Nonetheless, the definitive correlate of protection has not yet been identified. Until the protective immune responses are indentified, antibody and T-cell responses are being measured to provide confidence that the candidate vaccine is at least capable in eliciting an immune response, which is the basis of the mechanism of vaccines.

For some vaccines, a correlate of protection is used, which may not be the exact mechanism of action against the pathogen, but is correlated to the efficacy of the vaccine. An example is the hemagglutination inhibition test for influenza vaccines [6]. Obviously, the vaccine does not work by preventing the virus causing agglutination of hematocytes, but the number of antibodies that inhibit this phenomenon apparently is a good indicator of antibodies that block virus infection. Therefore, this should be regarded as a surrogate of protection [3]. Likewise, T cells are frequently enumerated by measuring IFN-γ secretion. The molecule IFN-γ itself does confer antiviral activity, for instance in inhibiting intracellular hepatitis B virus production [7], but this is not the only mechanism by which T cells combat infections. IFN-γ is mainly used because it is a marker of T-cell activation, and it is assumed that an activated T cell can also execute whatever actions are required to clear a given pathogen. However, it is becoming more and more evident that measuring IFN-γ alone is not sufficient; at least it has not provided the correlate of protection for many vaccines currently in development. In some cases, the immune parameter found to be a valid correlate of protection in one population may not prove to be as strongly correlating in another. CD4 T cells producing IFN-γ have been indicated to correlate with protection against tuberculosis disease in adults [8], while the cytokine profile of T cells of newborns after vaccination did not show correlation with protection against disease [9]. While antibody levels against Varicella-Zoster can serve as a measure of vaccine efficacy in adults, in a recent study in older subjects higher cell-mediated immunity levels were associated with reduced herpes zoster disease severity, while antibody levels were associated with more severe disease [10].

It is plausible that multiple immune parameters are required for protection against infection or disease. It is generally thought that antibodies can prevent initial infection, whereas T cells are required for clearing virus-infected cells and thus preventing disease. A combination of the two is thought to be required in response to vaccines against the major infectious diseases – malaria, tuberculosis and HIV. Furthermore, antibody titers are composed of a polyclonal antibody response, which is comprised of antibodies with various isotypes, concentrations, affinities, specificities and mechanisms of inhibition. Subsets or any combination of these antibody subsets may be responsible for protection. Virus neutralization is an obvious mechanism for which most vaccine developers are aiming, but binding antibodies could enhance opsonization and antibody-dependent cell-mediated cytotoxicity, which is more difficult to measure *in vitro*. Likewise, T-cell responses can be a mix of CD4 and CD8 T cells, effector cells, memory cells and other subsets, and the amount of T-cell markers all add to the complexity of the immune response. It is unlikely that a single T-cell marker is representative of a single mechanism of protection. However, amongst the many immune parameters, a single correlate or surrogate of protection may be found. Therefore, increasing numbers of parameters are combined in one analysis, resulting in huge amounts of data. Surface markers indicate the lineage of T cells, whereas various cytokine markers indicate the 'immunological diversity' of the activated T cells. Previous research has shown that multiparameter T-cell analysis can yield interesting information about the capacity of the immune response to influence disease progression in HIV-infected subjects [11,12].

For research purposes it seems that the more data gathered, the better for clinical development; however, more parameters to measure means more assays to control. It is not feasible to validate such assays for all different parameters that are of interest in exploratory studies, therefore a selection must be made as to what parameters are measured and to which level of quality these are controlled.

Our approach to solve this caveat is to develop and validate standard assays to show immunogenicity of the vaccine. In case of a malaria vaccine, we have selected a peptide-based antibody ELISA [13] and an IFN-γ Elispot assay. These assays have been thoroughly developed and validated, and although the predictive value of the results is not known, the data are reliable and reproducible, as evidenced by the validation results and assay performance as monitored by the quality control (QC) samples.

In addition, we have selected the more intricate T-cell intracellular cytokine staining assay as an exploratory assay that provides more detailed information on the phenotype of responding T cells.

Other malaria researchers have chosen to use functional assays, such as parasite inhibition assays, for the monitoring of the vaccine-induced response against the pathogen. In principle, functional tests more closely resemble the *in vivo* situation than the binding antibody ELISAs as they require recognition of the protein expressed in its most native form: on the sporozoites. However, functional assays are notoriously difficult to develop into a sufficiently robust set up. In addition, such assays usually require live pathogens as critical reagents, which add to assay variability and require additional safety measures.

Reagent issues

Assay development requires reagents in order to explore the platform on which the assay should be set up, the optimization of the assay and its characterization to provide a basis for validation. The amount of thought and effort that goes into preparing and securing these reagents, if they are not readily available, that is, if no gold standard is commercially available, is often underestimated.

Obtaining relevant reference or QCs can be a challenge. Assays for the detection of immune responses against a vaccine do not always have an established source of reference material, which bioanalytical assays for product potency or bioanalysis assays for pharmacokinetics do. For the latter assays, the drug that is under development or an international standard can be used for reference. In addition, in bioanalytical assays, the product is detected in buffer or spiked in serum, as are the positive QC samples, while a negative control sample consists of buffer or serum matrix. For development of vaccine immunogenicity assays, reference and control samples often have to be obtained from naturally infected donors, or in the absence thereof, need to be specifically created.

Reagents for serology

When we started development of a *Plasmodium falciparum* circumsporozoite (CS)-specific ELISA, serum with reactivity to the protein was sought to use as a reference in this semi-quantitative assay [13]. Through collaborations with other groups working on malaria CS-based vaccines, we could acquire serum of a CS vaccinated individual with high reactivity against the CS antigen. Alternatively, when developing a vaccine based on an antigen that is not yet in clinical phase, reactive serum has to be acquired from humans in regions where the pathogen is endemic. Preferably, the reference should be similar to the test samples. Although it has not been extensively studied, antibodies in serum from malaria-exposed individuals in endemic countries may be different to those of subjects that received one or two vaccinations. The presentation of antigen in a natural infection can be different from the antigen presentation in a vaccine, due to the use of inactivated pathogens, specific antigen epitopes and specific delivery systems. Repeated exposure to malaria, as is often the case in endemic countries, may lead to affinity maturation of the polyclonal response resulting in altered binding dynamics in antibody-binding assays.

Some blood borne infectious diseases do not allow for use of serum from exposed humans without extensive safety measures. Serum from Ebola or Marburg virus-exposed individuals, is extracted at biosafety level 4 and subsequently gamma irradiated to ensure viral inactivation. But even after inactivation, institutes may impose a required safety level that the laboratories are not equipped for.

In addition, in cases of the filoviruses Ebola and Marburg, outbreaks are unpredictable, both in location and timing, and are generally fairly limited in size. Although some serum can be acquired from the center of disease control, the nature of the outbreaks make the availability of reactive serum specific for Ebola and/or Marburg too rare for adequate assay setup and validation, let alone use as QC samples during assay runs.

In case of development of vaccine against pandemic influenza, a hemagglutination inhibition assay is performed as the generally considered surrogate of protection. This assay requires live virus that is subject to BSL3 safety level, which is only available in specialized institutes.

In cases where reactive serum for any reason is not accessible, other methods have to be employed to obtain suitable assay reagents that can serve as reference or QC samples.

Serum with reactivity to the vaccine of interest could be raised in experimental animals, however, the species specificity of the secondary/detection antibody does not allow for a direct comparison of performance of human samples in the assay. Alternatively, monoclonal

Key Term

Peripheral blood mononuclear cells: The (mononuclear) cells that are isolated from blood using ficoll and contain the major cell lineages of the immune system such as T and B cells.

antibodies may be raised against the vaccine of interest. However, dose–response curves of monoclonal antibodies may not be similar to the polyclonal nature of the vaccine-elicited immune response, which consists of many different clones of binding antibodies, each with their own specific isotype, specificity and affinity. The extent to which this can be a problem for assay validation and subsequent clinical sample analysis is discussed later. Registration of a vaccine against filoviruses such as Ebola and Marburg is subject to the animal rule, since challenge assays or field studies are not achievable. Part of the registration requirement is a predictive animal model, such as nonhuman primates, which can be bridged to the human immune response. In ligand-binding assays (LBAs), the secondary and/or detection antibody will be species-specific and even if the antibody is crossreactive, the affinity for different species will be different, preventing a direct comparison of the level of animal and human responses. To circumvent this, we propose to use a competition ELISA, in which both human and nonhuman primate titers are directly compared, based on their ability to compete with a specific serum from a tertiary species.

In addition to the specific aspects of acquiring reactive serum for reference and QC purposes, obtaining proper negative samples may also prove to be a challenge in certain cases.

When developing a vaccine against seasonal influenza, many sera may inhibit some level of reactogenicity due to prior exposure. The seasonal nature of the vaccine may pose problems of its own. Characterized and secured negative sera for one particular strain may not be negative for another strain, leading to renewed assay development and validation.

■ Reagents for cellular immunity assays

The strategies outlined above are suggestions for serology assays, detecting antigen-specific antibodies in (usually) serum. When it comes to determining the cellular immune response, there are far less alternatives or work-around strategies.

When developing an IFN-γ Elispot for the detection of malaria CS-specific T cells, no large batches of reactive **peripheral blood mononuclear cells** (PBMCs) were available to support the assay development and validation. Development was started using large batches of PBMCs isolated from healthy donors and a peptide pool used as a positive control for peptide stimulation, containing 38 dominant epitopes of CMV, Epstein–Barr virus and influenza virus (CEF peptides). Most donors in Europe and the USA would be exposed to at least one of these viruses, generating large numbers of responding donors from which great numbers of PBMC could be acquired. The assumption was made that the assay dynamics of a peptide response, whether it is against the CEF pool or the malaria CS peptide pool, would be similar and therefore CEF could be used to develop the assay and perform the assay validation. A successful validation leads to an assay-specific procedure that can be used for the detection of T cells reactive against different pools of peptides. A proof-of-concept assay that confirms the ability to detect CS-specific T cells was performed at a later stage, when reactive PBMCs were available.

This approach has also been employed for the generation of QC samples. Although not commonly done in T-cell assays, we wanted to be able to monitor the performance of the assay, just as we do routinely with serological assays. To generate PBMC batches that could serve as QC samples and determine the acceptance of each assay run, we isolated large batches of PBMCs from buffy coats obtained from blood banks. Buffy coats can supply up to 100 aliquots of PBMCs that can be frozen and stored in liquid nitrogen. The reactogenicity level against CEF peptides of each batch was characterized during validation of the assay in at least six assays to determine the nominal response and the range around the nominal response, which is considered acceptable for a successful assay run. Using these PBMC samples as QC samples throughout validation and clinical sample analysis, we are able to accept assay runs according to preset acceptance criteria, to monitor assay performance in time, and to train and qualify operators, as we do for LBAs.

■ Reagent & sample stability

The development of a vaccine from discovery to the market is a long process that on average requires 15 years. To support clinical trials with validated immunogenicity assays for this time and during large Phase IIb and III field trials, requires large amounts of reagents or regular bridging between lot numbers. Securing large quantities of reactive serum for reference and QC samples or regular bridging thereof, requires a considerable amount of effort. The undeniable benefit of a large batch of QC samples is the ability to trend assay performance over time. We have monitored the activity of QC serum for

many years and have found it to be very stable. We assume, therefore, that antibody binding and functionality as determined in neutralization assays is very stable if kept at <-65°C, which may even be extended to several decades. Trending these QC sera does not only provide information on the stability of the serum itself, but also on the complete assay performance and therefore on its critical reagents, such as cells and viruses, which may be more prone to variation.

Control over live biologics such as cells and viruses requires special consideration. As cells are passaged, genetic variation may accumulate with age, which might result in deviating responses when using increasing passage numbers. Cell banking at a relative low passage number, in addition to characterization of the upper limit of acceptable cell passage in the assay, is therefore recommended. The way of cell culture should be standardized as much as possible to prevent selection of sublineages when cells are stressed during low- or high-density culturing.

When assessing neutralizing antibodies in a functional assay, management of virus stocks is essential. Handling of viruses should be standardized, since viruses may be sensitive to temperature fluctuations, which could affect their infectiveness and, therefore, the assay results.

When a new virus strain needs to be introduced, simply substituting the old virus for the new strain and thereby considering the assay to be bridged does not suffice. In case of the adenovirus neutralization assay [14], the mechanism of infection needs to be considered. Different serotypes of adenovirus use different receptors for infection. The efficiency of virus infection is subject to the virus's preferred receptor being present on the reporter cell line. Although adenoviruses can very often infect through alternative receptors, they may do so with less efficiency. A 'virus-particle-to-cell' ratio titration is therefore recommended to assure a virus-to-cell ratio within the linear part of the response curve.

The microneutralization assay for influenza that was developed at our department assesses the inhibition of virus infection by neutralizing antibody response against the specific HA subtype on the virus. Virus infection is detected by measuring intracellular viral nucleoprotein, using nucleoprotein-specific detection antibody. This would lead to the assumption that in case of an influenza strain switch, exchanging the virus strain to be neutralized in the assay is sufficient. However, although the receptor for infection is the same, infection dynamics and interaction with HA on the virus may differ, resulting in the need for at least some development focusing on duration of incubation, infection titers and infection temperatures.

■ Sample control: storage & handling

Assuring proper collection and preparation of samples for analysis starts before initiation of the clinical study. The preparation of serum from blood samples is relatively simple and can be done without intricate equipment or extensively trained personnel. Once serum is extracted it can be frozen and kept stable for extended periods of time from -20 to -80°C. Relatively simple exchange of samples between clinical sites and laboratories can be done by shipment on dry ice, which can also be supplemented during transport, should the shipment be delayed.

For assessment of cellular responses, viable PBMCs are required to be stimulated directly in whole-blood assays or used freshly isolated. In most cases, however, the analyzing laboratories will not be sufficiently close to the clinical sites to allow this approach. It has been shown that PBMCs can be frozen without loss of viability or, more importantly, reactogenicity [15]. The proper isolation and freezing of PBMCs requires specialized equipment and highly skilled personnel, in addition to a well-guarded cold chain as the frozen PBMCs are very sensitive to temperature fluctuations, even at subzero temperatures. We have found, as others have noted, that laboratory personnel at clinical sites need to be sufficiently trained in handling blood samples and isolating and freezing of PBMCs [16]. In addition, PBMCs should be gradually frozen to -80°C with a subsequent move to liquid nitrogen storage within a few days. Freezing at higher temperatures or prolonged storage at -80°C will lead to a decrease in percentage-recovered viable cells and subsequent functionality in cellular assays [10]. Since in most cases shipment of PBMC will be required before analysis, ensuring proper cold chain management remains essential. PBMC should be shipped in specialized containers (dry shippers) saturated with liquid nitrogen [17]. It is recommended to verify the proficiency of laboratory personnel and cold chain management before studies start. Preferably, this would be done by conducting a dry run, during which PBMCs are isolated from blood by the clinical site, frozen, stored and shipped to the analysis laboratory. The PBMC quality is then evaluated by assessing viability, recovery and PBMC performance in control assays according to preset criteria.

This will ensure the qualification of laboratory personnel, the quality of the reagents and the cold chain, including shipment logistics, for the purpose of generating high-quality PBMCs once the study starts.

Guidelines

Assay development and validation is an essential part of drug development. Therefore, regulatory expectations on the rigidity of assays are high and comprehensively described in guidelines and White Papers. However, the scope of existing guidelines does not cover the combination of LBA and cell-based assay validation for vaccine potency tests in clinical samples. Several guidelines exist that can be applied to vaccines, such as US FDA and ICH guidelines on bioanalytical method validation, but these are aimed at product characterization and release tests, and are mainly based on experience with analytical assays such as LC–MS [18,19]. Additional White Papers from Findlay *et al.*, DeSilva *et al.* and Viswanathan *et al.* [20–22] provide further interpretation of the guidelines on how to apply the guidelines on LBA, but not yet for cell-based assays. Guidelines for assay validation applicable to clinical samples – including LBAs – are recently provided by the new European Medicines Agency guideline on validation of bioanalytical methods [23]. However, these are aimed specifically at pharmacokinetic bioanalysis, not at immunogenicity. Requirements for immunogenicity assays are described in several guidelines from the FDA, European Medicines Agency and White Papers [24,25], but these are specifically meant for unwanted immunogenicity of biotherapeutics instead of wanted immunogenicity of vaccines. All of these guidelines provide valuable guidance and considerations that can be used for clinical vaccine assays, but do not completely cover the specific application of vaccine immunogenicity tests. As mentioned in the introduction, vaccine assays could be categorized as a subset of biomarker assays. Although no official guidelines have been issued by regulatory agencies, several White Papers and conference reports do provide guidance on biomarker assay development, the 'fit-for-purpose' model [26] being the most relevant. Still, in our opinion, vaccine immunogenicity assays require yet another different approach because of several differences in purpose of the assay and especially the 'analyte' to be measured.

The intended purpose of the assay is the main driver for the scope of validation, and results in the main differences between potency release tests, as well as clinical vaccine assays. The differences between these two, in addition to bioanalysis tests, protein immunogenicity test and even biomarker tests make it complicated to select appropriate guidelines for clinical vaccine assays. The main differences are listed in Table 1.

The main difference between immunogenicity for vaccines versus biotherapeutics is the purpose of the assay: wanted versus unwanted immune responses. The main attribute of the unwanted immunogenicity test is the sensitivity, at which an unwanted event can be detected [27]. Whereas vaccines are expected to induce sufficiently high levels of immunity in order to protect against disease, and low levels of immunity are not considered to be relevant, unwanted immunogenicity is required to be detected at as low as possible levels in order not to miss any subjects that suffer from unwanted immunogenicity. Only after detection of positive subjects, is characterization of the antidrug antibody response is performed, and this usually includes a quantitation of the level of immunogenicity. Although the immunogenicity assays can be used in a (semi-) quantitative manner, the initial purpose of the assay is to qualitatively determine the presence of antidrug antibodies. Therefore, unwanted immunogenicity tests are designed as qualitative assays optimized for sensitivity, and validation focuses on the LOD or cut point [28]. For vaccines, it is important to know the number of responders. The relevant cut point of a responder is not necessarily the LOD, but is the level of immune response at which an individual is likely to be protected. Since this correlate of protection is not always known, vaccine assays are generally designed as quantitative assays to be able to measure the level of immune response over a large biological response range. This is the major common denominator between vaccine immunogenicity tests and biomarker tests. Both assays are designed to measure a biological response that is supposed to predict the outcome of the treatment.

The main reason that biomarker recommendations for assays are not directly applicable to vaccine immunogenicity tests is the nature of the analyte. Immune responses are complex biological processes that involve many different cell types and molecules. Although many immunological assays are designed to measure a specific part of an immune response, it is rarely a single molecule. Biomarker assays generally target a single molecule. Despite the fact that this molecule can exist in different states: bound versus free, polymorphisms, isotypes and post-translational

modification forms such as phosphorylation or glycosylation, the basic molecule can be described in a chemical notation or in an amino acid sequence. Even in the most basic immunological assay, such as ELISA, the resulting dose response is the sum of countless different antibodies with different sequences, affinities and concentrations. It is obvious that cellular immune responses have the same polyclonal complexity, where even cells originating from the same clone may be different in protein expression patterns and behavior.

Another level of complexity is added by the possibility of measuring different parameters of the analyte. Not only can the number of antibodies and cells be measured, but also the function and phenotype can be of interest. T cells also exhibit a continuously increasing array of characteristics that can be measured, such as surface markers and cytokine production. Whereas the T-cell Elispot assay, originally developed to detect antibody-secreting B cells [29], is based on the detection of IFN-γ as a single cytokine [30], ongoing development of the intracellular cytokine staining has resulted in the possibility of detecting multiple cytokines and phenotypic markers in one assay [11]. Virus-specific T cells can secrete the cytokine IFN-γ, but also IL-2, TNF-α, or a combination of different cytokines and other functional markers. Enumeration of T cells by IFN-γ secretion in two different subjects could result in similar T-cell numbers, but because the phenotype and expression of other cytokines could be different, the same number of IFN-γ-secreting T cells could be protective in one subject, but not in the other. Our strategy to handle this is that we will use one parameter (e.g., IFN-γ) as a standard read-out in a validated assay and other parameters, such as additional cytokines and cell phenotype, will be investigated in an exploratory assay. The additional information from exploratory assays should ultimately result in better understanding of the immune response and the correlate of protection. Once the immune response or combination of immune parameters that confer protection has been identified, it can be adopted as a standard assay and, if feasible, validation may be performed on the selected parameters.

The polyclonal nature of the analyte has consequences for some validation parameters, for instance parallelism. Parallelism is an important parameter in potency release tests and assays to determine drug concentrations in biological fluids. The dose–response curves of the samples need to be parallel to the reference curve in order to accurately determine

Table 1. Assay categories and characteristics.

Assay categories	Scope	Quantitative/ qualitative	Matrix	Reference	Analyte	Parameters/ measurement	Guidelines
Immunogenicity for vaccines	Wanted immunogenicity	Quantitative	Serum	True reference nonexistent	Polyclonal response	Multidimensional/sequential responses possible	None specific
Bioanalytical assays	Product potency	Quantitative	Formulation buffer	IS or drug	Monovalent analyte	Single parameter	ICH/US FDA/European Medicines Agency
Immunogenicity for biologics	Unwanted immunogenicity	Qualitative	Serum	True reference nonexistent	Polyclonal response	Multidimensional/sequential responses possible	European Medicines Agency/FDA/White Papers
Biomarker assays	Biological response	Quantitative	Serum/ biological fluids	True reference possible but difficult	Monovalent/ oligovalent analyte†	Multidimensional/sequential responses possible	White Papers
Bioanalysis	Pharmacokinetics	Quantitative	Serum	Drug	Monovalent analyte	Single parameter	European Medicines Agency/White Papers

†*Monovalent + isoforms, glycosylations.*
IS: International standard.

the concentration, independent of the dilution of the sample. Nonparallelism would lead to different results if samples are measured in the higher versus lower range of the reference curve. In principle, serum samples from different subjects – although treated with the same vaccine – are different in clonal composition, resulting in different dose–response curves. Therefore, parallelism between samples and a reference serum cannot be expected. In some immunogenicity assays, we use complete titration of the serum sample and in others we use two consecutive dilutions, which can be used to get an indication of parallelism. In practice, we have seen that most antibody responses are quite parallel after all, but there are individual exceptions. The question is what to do with the lack of parallelism, and how to interpret the data. Lee *et al.* suggested that a substantial departure from parallelism would invalidate the use of a reference standard, but a tolerable degree of nonparallelism could be acceptable [26]. We agree with this suggestion, although it should be noted that using dilution titers instead does not solve the problem of nonparallelism. Titers may be just as skewed due to differences in affinity and concentration as with using a reference curve.

The potential consequence of nonparallelism is that it may be more difficult to correlate the immune parameter with protection. A subject with a low titer of high-affinity antibodies may be protected against infection, whereas a subject with a high titer of low-affinity antibodies may not be protected. This would result in a less strict cut point for protection.

In general, the intrinsic assay variation of immunological assays is relatively large compared with analytical assays based on LC–MS, or even LBAs, because of the use of biological matrices, cells and/or virus. The polyclonal composition and multidimensional nature of the immune response also influences the variability of vaccine immunogenicity studies. Individual samples may be measured at a sufficient level of precision, but because the composition is different between different subjects, the protective capacity of the immune response may be different, despite similar reportable values in the same assay.

Because immunological responses, such as biomarkers consist of endogenous analytes, a background response or pre-existing immunity can be observed in normal healthy subjects. It is difficult, if not impossible, to definitely determine that a given donor is negative for the immune response of interest. There may be low antibody titers present due to previous exposure to the antigen, or antibodies to another pathogen may be crossreactive towards the antigen. It seems fair to assume that in a healthy western population of donors, no seropositive subjects will be found against, for instance, the Ebola virus. Still, the possibility of crossreactivity weakens this assumption. Indeed, we have found a malaria-specific T-cell response in a healthy Caucasian donor, who had never contracted the disease and who had never left Europe. The consequence is that a population of truly negative samples is difficult to obtain, and this hampers the determination of the LOD. In fact, it could be possible that the LOD we determine based on presumed negative samples is above the LOQ of the assay. Our approach to deal with this depends on the assay. For a T-cell Elispot, we designated the LOQ as the sensitivity cut point of the assay. For an adenovirus neutralization assay we have adopted the first dilution (1/16) as the technical LOD of the assay. A more pragmatic argument is that for immunogenicity assays for vaccines, we are not interested in the exact definition of the detection limit, but more in the protective level of immunity.

Future perspective

Challenges for immunogenicity assays for vaccines comprise the lack of information on the correlate of protection, the difficulty of obtaining reagents and references, and the lack of validation guidelines.

The first challenge actually makes the development of vaccine immunogenicity tests scientifically interesting. The search for effective vaccines and the relevant immune response are closely linked, and receive ample attention from researchers from academia, government institutions and industry. For clinical development of new vaccine candidates it would be more cost effective and time efficient if, after some time of exploratory research, the correlates of protection are defined, so that clinical laboratories can focus on the selected relevant parameters and use fully validated immunological assays. However, the current trend is not a convergence towards less, but actually a divergence towards more and more parameters. Using gene-expression arrays, the number of parameters have increased enormously, and in fact have proven to be highly valuable in identifying correlates of protection, for instance, for yellow fever [31].

Despite the lack of vaccine dedicated guidelines, developers of vaccines have been able to

set up and validate assays to support clinical development. However, since regulatory agencies are increasing their expectations and assay technologies are becoming increasingly more complicated, we argue that a centralized guidance would be beneficial. Many of the existing White Papers have emerged from working groups composed of different representatives from industry and regulatory agencies. It would be beneficial to have such platforms available dedicated to immunogenicity for vaccines, in order to discuss the described issues, exchange solutions and set acceptable industry standards for validation of immunogenicity assays for vaccines.

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Executive summary

Background

- Immunogenicity assays for vaccines are a distinct category of biomarker assay.

Correlates of protection

- The choice for the most relevant assay that correlates with protection against infection or disease is hampered by the lack of established correlates.
- Immune responses are polyclonal and multidimensional, which complicates the investigation into correlates of protection and assay validation.

Reagent issues

- Reagents for immunogenicity of vaccines are not always readily available or involve hazardous pathogens, which makes assay development and validation challenging.

Guidelines

- Existing guidelines and White Papers do not adequately cover validation requirements for immunogenicity assays. International cooperation is required to set industry standards.

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Papers of special note have been highlighted as:
▪ of interest
▪▪ of considerable interest

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▪ **Clear overview of the terminology and definitions used in the biomarker field.**

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