Mass spectrometry-based degradomics analysis of toxoid vaccines

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SUMMARY, GENERAL DISCUSSION AND PROSPECTS

Summary

Since their discovery, toxoid vaccines have proven to be reliable, safe, and effective. Inactivation of toxins, such as diphtheria and tetanus toxin, with formaldehyde results in very heterogeneous modified toxin molecules, called toxoids. The complexity of toxoid vaccines is exacerbated by adsorption of the toxoid to colloidal aluminum salts, leading to a colloidal system with a heterogeneous size distribution. Most conventional techniques to analyze biologicals, such as ELISA, UV absorption spectroscopy and size-exclusion chromatography, are incompatible with turbid analytes. Moreover, the heterogeneity makes it difficult to analytically characterize every antigen species in the vaccine; the mean result of an assay may hide the presence of certain antigen species that could significantly contribute to the safety and efficacy of the vaccine. Historically, functional tests for measuring immunity and safety in animals have been used to mitigate these issues. Up until today, such animal tests are being employed as batch release assays to test both safety and potency [1,2]. Efforts to replace or reduce these *in vivo* assays have been made based on a so-called consistency approach [3,4]: if the consistency between batches can be proven by using *in vitro* assays, no –or fewer– additional *in vivo* assays would be required. In line with this approach, we aimed to develop a new *in vitro* assay mimicking *in vivo* antigen processing and degradation. The field studying proteases, their substrates and their products has been termed *degradomics* [5]. Because differences in antigen processing *in vivo* are associated with differences in immunogenicity [6-12], mapping the enzymatic proteolysis of antigen and the peptides that are formed in a degradomics assay could be a viable alternative to animal tests.

Although toxoids are heterogeneous mixtures of formaldehyde-modified protein molecules, a well-designed and consistent production process should result in products with consistent quality. *i.e.*, potency, safety and stability. Characterization of the detoxification process and the resulting changes in the protein can support the development of new assays and can be used to compare different products. In **chapter 2,** formaldehyde-induced chemical modifications of diphtheria toxoid were mapped by mass spectrometry. To overcome the issues associated with the toxoid's heterogeneity, a bottom-up proteomics approach was applied, *i.e.*, analyzing the enzymatically digested toxoid. The diphtheria toxin was prepared with regular or deuterated formaldehyde, which then allowed software tools to compile a list of peptides with formaldehyde adducts. After identification of the modified peptides,

the location of the formaldehyde-induced modifications in the protein was obtained. The NAD^+-binding cavity and the receptor-binding site, important for the toxin's toxicity, were found to be affected by formaldehyde. Additionally, an important CD4 T-cell epitope was unaffected. Many adducts were crosslinks between glycine (present in the inactivation matrix) and the toxoid. If the glycine used in our studies would be replaced by other amino acids (or other amine-containing chemicals in general), as is sometimes the case, this will result in a toxoid that is different on a molecular level. Therefore, care should be taken when choosing (or changing) the matrix components. The heterogeneity of the toxoids makes it impossible to definitively identify a single formaldehyde modification responsible for loss of toxicity or potential loss of immunogenicity. Nevertheless, a thorough characterization of these products with an approach as presented in **chapter 2** could help to compare different products and assess their comparability.

The methods used in **chapter 2** can be applied for the identification of formaldehyde-induced modifications in other proteins as well. In **chapter 3** the effect of formaldehyde-induced modifications on the enzymatic proteolysis of bovine serum albumin, β-lactoglobulin and cytochrome c by cathepsin S is described. Contrary to our expectations, exposure to higher concentrations of formaldehyde made these model proteins more susceptible to proteolytic degradation. The most abundant chemical modifications could be mapped. Some specific modifications in cytochrome c –in a particular formaldehyde-glycine adduct– were found to be responsible for faster degradation of certain parts of the protein. However, even proteins subjected to formaldehyde in the absence of additional amino acids showed increased degradation overall. The differences in enzymatic degradation kinetics obtained after incubation with various concentrations of formaldehyde highlight the influence of the various aspects of the inactivation process, such as the importance of using a standardized amount of formaldehyde for antigen detoxification. Our newly developed degradomics assay could pick up these formaldehyde-induced changes in proteins of interest.

Unexpected mass increases of peptides derived from formaldehyde-treated cytochrome c were observed during the studies of **chapter 3**. **Chapter 4** focuses on the structural elucidation of these formaldehyde-induced modifications. Two proximate lysine residues were able to undergo (*i*) formaldehyde-induced deamination and formation of α,β-unsaturated aldehydes and methylation on the two lysine residues, and (*ii*) formaldehyde-induced methylation and

formylation of two adjacent lysine residues. These modifications result in intramolecular cross-links between two lysine residues in a single peptide, but also form cross-linked dimers and trimers. Knowledge of these potential formaldehyde-modifications in vaccine products could aid in the development and registration of new vaccines and contribute to the characterization of current vaccines.

Chapter 5 describes the application of the degradomics approach for the analysis of tetanus toxoids. Several tetanus toxoids were obtained from different manufacturers and subjected to elevated temperatures to simulate faulty batches. First, the stressed samples were subjected to cathepsin S digestion, and the kinetics of peptide formation was evaluated. Subsequently, three suitable stability-indicating peptides were selected and quantified after a fixed digestion time. Our improved degradomics assay could pick up temperature-induced changes in these products. Importantly, the method worked for both non-adsorbed and adsorbed tetanus toxoids. Therefore, analysis with our method is not restricted to intermediate (non-adsorbed) bulk toxoids but can also be used to assess the quality of the final (adsorbed) drug product. Our degradomics assay is both sensitive and precise, making it a good potential alternative for the current animal tests.

To be able to easily apply the degradomics assay to other antigens, the analysis of the kinetic profiles of all peptides originating from enzymatic degradation was further optimized in **chapter 6**. By using tandem mass tags (TMT), multiple samples could be compared to a pooled control sample: the *common reference*. This greatly reduced the analysis time and decreased assay variation, enabling easier implementation of our degradomics approach to other antigens and vaccines.

General discussion and prospects

The main objective of this thesis was to develop a new *in vitro* assay that can confirm product consistency and potentially can replace the current *in vivo* assays used for the batch release of toxoid vaccines. Our envisioned assay was inspired by the immune system, where changes in antigen degradation in antigen-presenting cells have been correlated with altered immune responses; slower degradation has been found to correlate to higher immunogenicity [6-12]. From this starting point several approaches were possible: antigen degradation by dendritic cells (DCs) has been studied (*i*) *in vivo* and (*ii*) *ex vivo* [9], (*iii*) in cell lines [8], in (*iv*) lysosomal extracts derived from DCs [8], and (*v*) in assays where only key enzymes and proteins are added [6,13]. For future routine use, assay robustness is key. With decreasing biological and immunological fidelity, the complexity of the assay also decreases. For instance, it is much easier to produce a recombinant enzyme in a consistent manner than it is to obtain lysosomal extracts from DCs in a consistent manner. Therefore, we opted to develop a degradomics assay based on recombinant human cathepsin S. Arguably, showing consistency with a precise method is more important for a vaccine release assay than showing a (slightly) more direct correlation with immunogenicity with an imprecise method.

An ideal *in vitro* assay would be a direct measure for a vaccine's immunogenicity. However, in reality this is not feasible for any single *in vitro* assay. Because of the complexity of the immune system, the currently implemented alternatives for certain immunological animal tests always focus on a specific aspect of the immune system. For example, the polio D-antigen ELISA release assay [14] is used to test polio vaccines based on antigenicity, whereas the intended effect of the vaccine is to induce a protective immune response (immunogenicity) and not to bind antibodies (antigenicity). Similarly, alternatives to rabbit pyrogen tests involve cell-based assays based on monocyte activation [15]: again the readout is related but not identical. The degradomics assay attempts to mimic antigen processing, which is an important step in inducing a CD4 T-cell response, but it is also limited in that it only covers a part of the T-cell response and not the –at least– equally important B-cell response needed for the production of protective antibodies. Moreover, the assay as presented in **chapter 5** does not include other important proteins involved in MHC-II epitope selection and processing, such as other proteases, HLA-DM (involved in peptide selection) or HLA cell surface receptors. These additional proteases can be added as demonstrated by Isamu Hartman *et al.*, but

this would severely increase the assay's complexity [13]. From a scientific point of view, it may be interesting to evaluate the correlation between the immunogenicity of a vaccine and the readout of our degradomics assay. Tetanus toxoids exposed to elevated temperatures have shown decreased potency in previous studies [16]. Indeed, the results described in **chapter 5** show enhanced enzymatic proteolysis upon heat treatment of tetanus toxoids, which supports the hypothesis that antigens that are more prone to proteolysis are less immunogenic. In general, tetanus and diphtheria toxoids were found to be very protease resistant, especially when compared to the rapid enzymatic degradation after the toxoids were exposed to elevated temperatures. Formaldehyde-treatment of model proteins resulted in faster proteolytic degradation, so it would be interesting to both determine the immunogenicity of model proteins and their formaldehyde-treated counterparts. If proteolysis correlates with immunogenicity, the formaldehyde-treated proteins will not be more immunogenic compared to their native model proteins. Furthermore, it would be interesting to determine the effect formaldehyde-treatment of diphtheria and tetanus toxin has on their enzymatic degradation. Previous research has shown that formaldehyde-treatment enhances the immunogenicity of diphtheria toxoid compared to diphtheria toxin [17], so slower degradation would be expected despite our findings with model proteins. To find a stronger correlation between protease resistance and immunogenicity, antigens should be altered in such a way that the protein's B-cell epitopes are minimally affected but antigen proteolysis is either enhanced or decreased, similar to the minor changes of the antigens compared in a study by Egger *et al.* [8]. Analysis of a similar set of vaccine antigens in an *in vivo* immunogenicity study could then be used to confirm the correlation. However, from an ethical point of view it seems counterintuitive to perform an animal experiment with the goal to reduce the number of animal tests. Moreover, from a quality control point-of-view, showing product consistency –rather than demonstrating a correlation with *in vivo* immunogenicity– is key. There are always more insights to be gained from additional animal tests, but their use should be limited if they are not strictly necessary to reach the predefined study objectives, as is the case in this thesis.

Compared to other *in vitro* alternatives that are being developed or have been developed, the degradomics approach has several advantages. Most other assays, such as physicochemical techniques [17,18], ELISAs [19] and cell-based assays [20-23], require biological reference samples for comparison. Limited shelf life and batch-to-batch consistency of such standards are

important considerations, although, for instance, various toxoid standards are available from the National Institute for Biological Standards and Control (NIBSC). If the formation of specific peptides is analyzed with a degradomics analysis, absolute quantification (*i.e.*, moles per antigen quantity at a given reaction time) can be achieved with the help of synthetic internal standards, and the assay does not rely on biological reference samples. Production, characterization and validation of these standards is much easier than the use of biological reference standards, and should facilitate technology transfer to labs world-wide. Another advantage of the degradomics assay is that it evaluates specific parts of the protein and can identify changes in a small subset of proteins. In other assays, such as physicochemical methods, the average response of the antigen is measured. Small changes in large proteins could remain unnoticed because they might have little influence on the average response. Analysis of parts of the protein that are particularly sensitive to inconsistency (including production- and storage-induced changes) could result in a more sensitive assay. Further advantages include the orthogonal nature of the assay compared to the other *in vitro* alternatives and the very limited amount of sample required for mass-spectrometric analysis. The use of mass spectrometry, however, does come with drawbacks; it requires relatively expensive specialized equipment and specialized personnel. Moreover, the wealth of data generated makes it challenging to evaluate everything, and the much-needed focus on a small subset of the data could introduce bias, particularly when setting up the assay for a new antigen. Finally, the degradomics assay can be used to test antigens in the presence of aluminum salts, so the final drug product can be assayed, something that has been challenging for other assays. Overall, the complementarity with other assays makes the degradomics approach a very suitable candidate for a panel consisting of multiple tests, which could be used for both stability and release assays, because its pros and cons are complementary to other *in vitro* tests and different parameters of the product are analyzed. For instance, when the results of a degradomics assay are combined with those of an antigenicity assay such as an ELISA, both correlations to the important CD4 response (antigen processing) and binding of the B-cell to the antigen (and antigen processing in the B-cell itself) are covered.

In this thesis, a proof of principle for an *in vitro* degradomics approach for quality control of toxoid vaccines has been established. However, several other research questions, which could be answered in subsequent studies, remain. These topics can be divided into research questions related to the vaccine(s) and those related to the assay itself. For instance, the

studies in **chapter 3**, which addressed the effects of formaldehyde-inactivation on model proteins, could be followed up by studies on toxin inactivation and its effect on enzymatic proteolysis. Formaldehyde inactivation is a key step in toxoid vaccine production processes, both for safety and efficacy, so it would be ideal if our assay could pick up unwanted changes in the inactivation process. The working degradomics assay is described for tetanus toxoid in **chapter 5**, and general proof of principle is described for diphtheria toxoid in **chapter 6**. A subsequent step would be to show that the assay works for an actual (combination) vaccine containing all excipients, adjuvant and other antigens. Moreover, the assay could be further optimized to include the commonly included polio and pertussis antigens, so that all antigens in the combination vaccine can be assayed at once. This raises the question whether the degradomics assay is capable of evaluating more complicated antigens, such as tetanus toxoid-haemophilus influenzae type b polysaccharide conjugates. It seems likely that the polysaccharide-conjugated tetanus toxoid will react similarly to the unconjugated toxoid, *i.e.*, exposure to higher temperatures leads to faster enzymatic degradation. However, it remains to be seen if the degradomics assay will provide useful insights, since the polysaccharide contains the relevant B-cell epitopes, although an adequate CD4 response to the carrier protein is important for long term efficacy [24]. Further research into the degradomics assay itself may shed more light on the usefulness of this assay for conjugate vaccines. For more complex antigens such as live attenuated vaccines and vector-based vaccines, mimicking the MHC-II processing pathway seems illogical. Other assays (such as virus titration) are more suitable for the analysis of those products. For further studies it would be interesting to evaluate the degradation kinetics of the antigens when treated with other proteases, such as trypsin. It is likely that for antigens that are not treated with formaldehyde, which affects the lysine and arginine residues needed for trypsin digestion, similar kinetic trends will be observed when using trypsin (or other proteases) instead of cathepsin S. Finally, the discovery of novel formaldehyde modifications described in **chapter 4** suggests that several related undiscovered modifications could be present in formaldehyde-treated antigens. Identification and characterization of these products in real vaccines would be another important research topic and could aid in the development and registration of new formaldehyde-inactivated vaccines, as well as support the replacement of animal tests in current vaccines.

Altogether, besides the additional knowledge about formaldehyde-adducts (**chapters 2, 3** and **4**), the degradomics approach has resulted in a sensitive assay that may be able to pick

up subtle changes in the protein folding of antigens used in vaccines. Actual implementation of the degradomics assay described in this thesis and those proposed by others for the batch-release of vaccines requires further validation by vaccine manufacturers and cooperation by regulatory agencies. However, all parties involved should have sufficient incentives (*e.g.*, reduction in assay variation, reduction in costs) to ensure that further reduction and replacement of animal tests is achieved in the coming years. Moreover, the data presented in this thesis could provide useful insights into the characteristics of new vaccines and provide alternatives to animal tests before *in vivo* release tests are implemented.

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