REVIEW

BIOTECHNOLOGY and BIOENGINEERING

Host Cell Proteins in Biologics Development: Identification, Quantitation and Risk Assessment

Xing Wang, Alan K. Hunter, Ned M. Mozier

Pfizer, Inc., Global Biologics, 700 Chesterfield Parkway West, Chesterfield, Missouri 63017; telephone: 636-247-6349; fax: 636-247-5351; e-mail: ned.m.mozier@pfizer.com

Received 7 November 2008; revision received 6 February 2009; accepted 12 February 2009

Published online 22 April 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22304

ABSTRACT: Host cell proteins (HCPs) are those produced or encoded by the organisms and unrelated to the intended recombinant product. Some are necessary for growth, survival, and normal cellular processing whereas others may be non-essential, simply carried along as baggage. Like the recombinant product, HCPs may also be modified by the host with a number of post-translational modifications. Regardless of the utility, or lack thereof, HCPs are undesirable in the final drug substance. Though commonly present in small quantities (parts per million expressed as nanograms per milligrams of the intended recombinant protein) much effort and cost is expended by industry to remove them. The purpose of this review is to summarize what is of relevance in regards to the biology, the impact of genomics and proteomics on HCP evaluation, the regulatory expectations, analytical approaches, and various methodologies to remove HCPs with bioprocessing. Historical data, bioinformatics approaches and industrial case study examples are provided. Finally, a proposal for a risk assessment tool is provided which brings these facets together and proposes a means for manufacturers to classify and organize a control strategy leading to meaningful product specifications.

Biotechnol. Bioeng. 2009;103: 446–458.

KEYWORDS: host cell proteins; ELISA; Western blot; proteomics; genomics; bioinformatics

Introduction

Host cell proteins (HCPs) are unique to their respective hosts (types and strains) used for biologics production. The composition and abundance of HCPs present in various steps of manufacturing processes and in the final drug substance depend on many factors. First and foremost, the composition is closely associated with the host expression system itself. For example, *E. coli* has ~4,300 genes (Blattner et al., 1997), whereas mammalian cells such as Chinese Hamster Ovary (CHO) and a commonly used mouse myeloma cell line (NS0) have about 30,000 genes (Gibbs et al., 2004; Waterston et al., 2002). Secondly, the composition of HCPs is related to the manner in which the biologic of interest is expressed. For example, recombinant proteins produced in *E. coli* may be expressed directly in the cytoplasm or secreted into the periplasm, whereas in mammalian cells it may be secreted into the culture medium. Alternatively, in *E. coli*, the protein of interest may be engineered to be deposited as inclusion bodies in the cytoplasm or periplasm. Each of the different expression modalities and techniques can produce different populations of HCPs (Hart et al., 1990; Rinas and Bailey, 1992; Rinas et al., 1993; Veeraragavan, 1989). Thirdly, the HCP composition at various steps of the manufacturing processes is related to the purification process itself (Hart et al., 1990; Rinas et al., 1993). Since the primary recovery and purification steps of a biologic manufacturing process each rely on a limited subset of physiochemical properties (e.g., ion exchange takes advantage of charge differences), a subpopulation of HCPs will normally co-purify with the protein of interest regardless of the purification process that is employed. The advantages of specific, high affinity purification techniques (e.g., Protein A capture for monoclonal antibodies) are well known, as they typically enable the removal of a vast majority of HCPs. Finally, the composition of HCPs depends on the biologic molecule being expressed (Hunter et al., 2009; Shukla et al., 2007, 2008). The physiochemical properties of the intended recombinant protein (charge, hydrophobicity, structure, etc.) influence the HCPs present at various stages in the bioprocess due to co-purification of proteins with similar attributes as well as non-specific associations. These factors can be quite difficult to predict a priori and are often learned only by testing during process development.

Characterization is important because HCPs carry potential clinical safety risks in addition to those that might be related to the intended recombinant protein itself. These risks are difficult to evaluate since pre-clinical models are rarely informative and case studies in human studies even rarer (an example is provided at the end of this article).

Correspondence to: N.M. Mozier

The foreign or ''non-self'' nature of HCP derived from non-human expression systems suggests that almost any individual HCP has the potential to elicit an immune response in humans, although the magnitude and nature of any response will depend on the composition and amount of foreign components introduced and the status of the human immune system (Janeway et al., 2005). On the other hand, current bioprocess technology cannot produce absolutely HCP-free material from a living organism. It will be obvious to the reader that improvements in testing sensitivity will reveal trace HCPs in drugs previously believed to have ''undetectable'' levels of HCPs. The absence of human data and the nearly infinite variety of HCPs potentially present make this a challenging problem. Industry addresses this risk by diligent method development and application of learnings from the few case studies available. The use of multiple and orthogonal technologies, conventional and unique, to detect and evaluate the virtually unlimited variety of HCPs that might occur during bioprocess development leads to the development of robust and well-controlled bioprocesses, which in turn minimize risk as new drugs are brought to market. The scope of this review is to focus on HCP analytical testing and its use in risk assessments for recombinant therapeutic proteins, other process-related impurities such as residual DNA and endotoxin will not be covered here.

Regulatory Aspects/Risk Analysis

The level of HCPs in biologics are most often expressed as nanogram HCPs per milligram of drug substance (ng/mg) or parts per million (ppm) determined by a quantitative Enzyme-linked ImmunoSorbent Assay (ELISA) or, in rare cases, Western blotting with anti-HCP antisera. The biotechnology industry currently uses a combination of ''commercial'' and ''customized'' ELISA assays for HCP quantitation (Champion et al., 2001; Krawitz et al., 2006). This terminology can vary from company to company and over time, but we use the term ''commercial'' to mean the publicly sold reagents (immunological reagents and standards) that are typically made as a mixture of a variety of strains and designed to encompass the range of HCPs that might be present in any expression system. The term ''customized'' is intended to mean a company-specific (usually proprietary) combination of reagents (immunological and standards) that are designed to be specific for that company's specific host organism. Regulatory expectations about which is suitable usually entail a risk assessment, the stage of product development (years may be required to develop a customized kit for a product), and the experience the sponsor has with other programs. The ultimate suitability of the HCP test is based on the results obtained both in detecting and quantifying the residual HCP levels in late stage or registration batches made at the commercial scale.

Because there is no universally available and accepted testing modality, and a generalized lack of standardization in the industry, there is currently not a single test or absolute control limits required by regulators during clinical trials and at registration. A recent report has indicated that the most likely range of HCPs in biologic products reviewed by FDA is 1–100 ppm (Champion et al., 2005). Many biotechnology companies are using this range as a guideline for process development and for setting HCP specifications. Essentially, the level of acceptable HCPs is reviewed on a case-by-case basis by the regulatory authorities. Helpful for industry is the guidelines published on this topic from European and United States agencies (EMEA, FDA) and the international conference on harmonization (ICH): (EMEA, 1997; FDA, 1997; ICH, 1999). The regulator perspective is summarized below, where factors for considerations are enumerated as follows:

1. Process capability.
2. Maximum dose (mg biologics/kg body weight).
3. Route of administration (subcutaneous (Subcut), intramuscular (IM), intravenous (IV), etc.).
4. Frequency of dosing (acute or chronic indications).
5. Pre-clinical and clinical data.

These parameters are useful to discuss risk and are most valuable for companies with significant history to draw upon. An example is provided in Figure 1 with some detail on the first factor, as the focus of this review is bioprocessing and analytical testing of material. The frequency of dosing and the route of administration are also useful risk assessments, particularly around potential for immunogenicity, and are discussed here in general terms, but a detailed discussion is beyond the scope of this review. Figure 1 is an example of how a manufacturer might assess process risk and develop a control and testing strategy. For example, microbial systems are generally at higher risk of HCP occurrence for the simple reason that the starting load of HCPs (relative to recombinant target protein) is greater. Within microbial systems there are a number of modes of expression which impact this starting load, whereas mammalian systems tend to have two main categories depending on whether the process starts with an affinity step such as Protein A capture. Overall, the severity of the risk of HCP contamination is valued the same in this example since, in the estimation of the authors, the higher reported incidence of microbial proteins causing immunological effects in humans (Ohmura et al., 1987; Ullenhag et al., 2001) is balanced by the risk that antibodies to HCPs from non-human mammalian cells in theory might then adversely react with similar endogenous human regulatory proteins. This rationale is discussed further in the next section. It should be noted that Figure 1 is an example of a process by which a manufacturer might internally assess processing risk and is illustrative of the factors to be assessed. The example given puts the risks into three main categories: severity, occurrence, and detectability, although others might increase the detail and prefer putting risk in quintiles or quartiles. In many cases the nature of the product, or

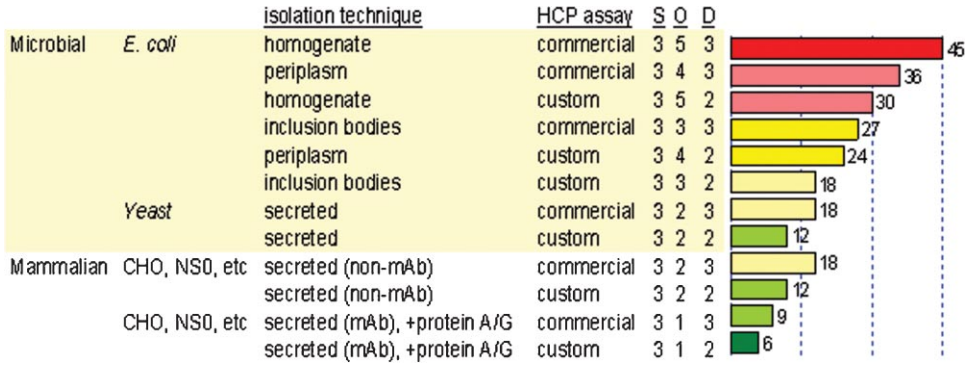

Figure 1. Risk analysis for HCP control during bioprocess development. S, severity; O, occurrence; and D, detectability. Risks for severity and detectability are valued on a scale of 1, 2, or 3 where 1 = least and 3 = most. The risk of occurrence has greater weighting and is on a scale of 1–5 (5 = most). The overall score is the product of multiplying these three factors together. Vertical dotted lines show the overall risks in groups in terciles where the highest risk category is shown in red and the least risk category is in green.

requirements for yield to meet commercial product volumes, will drive the selection of expression system and isolation procedures. These inherent demands are balanced by others that are controllable, such as the choice of assay. For example, Figure 1 shows that choosing a customized assay is a controllable means to reduce risk by improving detection sensitivity. This factor is also a valuable consideration when establishing control limits and specifications and should be part of the overall risk evaluation. In conclusion, the exercise of conducting a risk assessment, rather than the particular algorithm used, is where the value is realized.

The importance of HCP evaluations are best understood in the context of the overall development program for biotherapeutic agents. Risks include potential clinical side effects from the drug candidate as well as the failure to meet clinical endpoints (Nick, 2006). Because of an incomplete understanding of human physiology and the complex interactions of any drug candidate with the human body, many risk factors are impossible to predict and are often beyond control. HCPs, on the other hand, represent some degree of risk as unintended components in the drug product, but there are a number of analytical options for their detection and measurement. These measurement tools can therefore be employed to reduce such HCP-related risks, even beyond the custom versus commercial ELISA options evaluated in Figure 1. Given the high attrition rate during drug development, it is a practical matter to consider putting a comprehensive HCP program in place to help ensure that HCPs are evaluated properly and help mitigate the risk that they contribute to the failure of an otherwise promising therapeutic molecule.

The Biological and Immunological Consequences of HCPs

An understanding of the biological and immunological consequences of HCPs is worth considering when developing approaches for HCP evaluation because it illustrates the complexity of molecules that may be present. First of all, recent developments in genomics and proteomics indicate that the potential number of HCPs from a given biologic process could be very large. For example, *E. coli* has about 4,300 genes and because the corresponding gene products may often undergo unique post-translational modifications, this can greatly increase the total number and biochemical complexity of the HCP population associated with a given bioprocess. On the other hand, it is known that not all the genes are expressed, and that some genes are expressed at different times and under different conditions. In mammalian cells, the number of genes and protein modifications are significantly greater than that in prokaryotic cells. It has been reported that one protein was found to have more than 20 different modified forms (Godovac-Zimmermann and Brown, 2001). Examples of the types of modifications that can generate HCPs with different biochemical properties, and thus further complicate the HCP evaluation process, include glycosylations, phosphorylations, and truncations. Another aspect of HCP evaluation is the knowledge that the level of HCPs that may be produced has a very wide dynamic range. It has been reported that protein expression levels in eukaryotic cells can display more than a 6 order of magnitude range (Belov et al., 2001; Godovac-Zimmermann and Brown, 2001). This is a very important factor to consider when developing the HCP evaluation strategy because any analysis can only start with a limited amount of proteins (usually <1 mg total proteins), and that will limit the level of HCPs that can be detected. As will be discussed in Section 5.1, there are different approaches to increase the range for HCP detection and quantitation.

A central question in the study of HCPs is how to evaluate the risk to human health associated with their presence in drug product. For example, it is well known that the greater the difference between the encountered foreign molecule or organism, the greater the potential for a recognition by the mammalian immune system as not self

(i.e., immunogenicity; Janeway et al., 2005). Because the genomes from recombinant expression systems used to product biotherapeutics such as CHO, yeast and *E. coli* are different from that of humans (Lander et al., 2001; Venter et al., 2001), it is expected that at some level many if not most HCPs will induce an immune response in the human body. However, as mentioned earlier, the composition of HCPs in the final drug substance depends on many factors and it is difficult or impossible to design non-clinical or in vitro experiments to demonstrate which HCPs and at what level may confer risk to humans.

However, it is instructive to consider what information and/or techniques are available to better understand the relative risks. For example, it has previously been shown that proteins stimulating the immune-system sometimes can be assessed by measuring the release of inflammatory cytokines with whole blood assays from animals and humans (Groote et al., 1992; House, 2001; Meager, 2006). Figure 2 is an example of the authors' experience with a program where cytokine release assays are complimentary to HCP analyses using process-specific Western blotting. It can be seen that a reduction in HCPs achieved through process modification that is guided by the cytokine release assay is correlated with a reduction or elimination of the induction of Interleukin-6 in an ex vivo human whole blood assay using normal volunteers.

The potential health risk of HCPs can also be viewed from a different biological angle. It is known that some of the essential human regulatory proteins exist in the human body at ng/mL and are biologically active, examples being the cytokine and chemokine families (Anderson and Anderson, 2002). Recent genomic sequencing demonstrates that humans and mice share about 80% homology (Waterston et al., 2002). It is therefore theoretically possible that some HCPs derived from non-human mammalian cells could also function in the human body when administrated at relatively low levels, although reports of this have not been made. In such a case, antibodies raised to non-human mammalian HCPs could theoretically react with similar human analogues, thereby affecting normal function (although, again, to the author's knowledge reports of this risk have not been published). On the other hand, the amount of *E. coli*-derived HCP required to induce an immune response could be much smaller than that from mammalian CHO or NS0 cells since there is more evolutionarily divergence in the former (from humans).

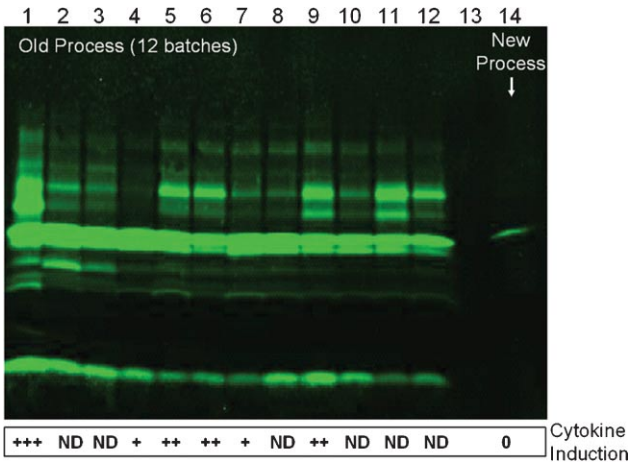

Figure 2. Western blotting (highly sensitive, samples = ppm) results of batches and reactions in human whole blood assays monitoring Interleukin-6 release. **Lane** 1–12, different batches of drug substance from a previous campaign, batch numbers were labeled in the first row. **Lane** 13, blank; **lane** 14, drug substance from the new process. Fifty microgram per lane of drug substance was loaded in a SDS–PAGE for protein separation. For cytokine-release activity measured in the whole blood assay, +++, strongest; ++, intermediate level response; +, weak response; ND, not determined; 0, no cytokine response.

Analytical Technology Overview

Many analytical technologies have been used for the detection, identification, quantitation, and risk assessment of HCPs (Briggs and Panfili, 1991; Eaton, 1995; Hoffman, 2000). It is expedient to select the right combination of technologies for any specific bioprocess, and most importantly, to understand the advantage and limitation of each technology so that an optimal strategy can be developed for a bioprocess-specific HCP evaluation. This section will give an overview of the different technologies and approaches mostly used in HCP detection and evaluation.

Separation and Visualization Methods for Host Cell Proteins

HCPs can be separated and visualized using many different techniques. The two most common approaches are protein staining and immune detection with Western blot. Before the advent of sensitive immunoassays, these were the norm and considered sufficient. In the protein staining method, the HCPs are first separated in a polyacrylamide one-dimensional or two-dimensional gel, fixed with acid (usually acetic acid), followed by staining. Various staining methods have been developed over the years; the most common methods include Coomassie Blue, silver staining, and Sypro Ruby staining (Speicher, 2008). The sensitivity of Coomassie Blue is in the range of 0.05–0.1 μg/band or 2D spot, whereas both silver and Sypro Ruby staining could have 1–5 ng/spot sensitivity. However, Coomassie can stain proteins differently, and silver staining sensitivity depends on the sulfhydryl and carboxyl groups in the protein, therefore, for proteins that have lower proportions of these groups, the silver staining sensitivity will be decreased. The recently developed Sypro Ruby or Sypro Orange fluorescence staining is independent of the composition of the protein; therefore different proteins can be visualized more evenly. Another advantage of Sypro Ruby staining is its wide dynamic range. It was estimated that Sypro Ruby staining

has about a 1,000-fold dynamic range whereas silver or Coomassie Blue staining only have 10- to 100-fold dynamic range (Speicher, 2008). A further advantage of fluorescence staining is that it is mass spectrometry friendly. Because HCP evaluation may eventually depend on the identification of the HCPs detected, it is important to have a staining approach that will make the further characterization possible. Perhaps the biggest downside of staining techniques is the lack of specificity such that the presence of the intended recombinant protein is overly abundant and obscures smaller bands. Detection methods require contrast for signal to noise and the enormous excess of the major band and its degraded forms can make it very difficult to see smaller entities such as HCPs present in trace quantities.

Western blot is an antibody-dependent detection method (Speicher, 2008) that has merit and still is a common tool. This method offers advantage in its sensitivity and specificity; sometimes as low as picograms of protein have been identified with Western blot. Samples containing the protein of interest will first be separated with either one-dimensional or two-dimensional gels, and transferred to a PVDF or nitrocellulose membrane. The membrane is first blocked with BSA or other proteins that will occupy the protein binding sites on the membrane, the primary antibodies raised against HCPs will be incubated with the membrane and formed complexes with the HCPs on the membrane. The HCP-antibody complex could be detected by either directly labeling the primary antibody with an enzyme like Horseradish Peroxidase (HRP) or a fluorescence molecule or detected indirectly with a labeled secondary antibody that specifically recognize the primary antibody (Speicher, 2008).

For non-IgG biologics, both the primary and secondary antibody labeling approaches are useful for analysis in the Western blot. For monoclonal antibodies expressed in CHO or NS0 cells, the direct labeling of the primary antibody is highly recommended. This is due to the fact that the human IgGs derived from CHO or NS0 and animal IgGs raised against HCPs are highly homologous, it is difficult to eliminate the cross-reaction of the secondary antibody toward the human IgGs, making the Western blot insensitive and non-specific. When only primary antibodies are used, after labeling they are specific to HCPs and cross-reactivity is minimal. It is important to generate an anti-HCP antibody from an HCP preparation that is free of IgG; as is described in the next section, parts per million (ppm) levels of IgG contamination in the HCP immunogen will jeopardize the Western blot and quantitative ELISA for HCP detection and quantitation.

Development of Immunoassays for HCP Quantitation

There have been several reports on the generation of anti-HCP antibodies and development of immunoassays (Anicetti et al., 1986; Dagouassat et al., 2001; Thalhamer and Freund, 1984; Zhu et al., 2005). It is worth noting that a high quality preparation of the HCPs to be used in anti-HCP antibody generation is critical to the success of the HCP program. Ideally, the HCPs present in the biologic manufacturing process are the most relevant, and should be used as immunogens for raising anti-HCP antibodies. However, it is practically very challenging if not impossible to prepare HCP using a process feed stream derived from the production cell line because the abundant therapeutic protein must be eliminated from the preparation with incredible fidelity (below a few ng in mg of HCPs). Therefore, most HCP antibodies are raised from immunogens prepared from null cell lines (obtained from the same cell lines but without the genes necessary to express the biologic product of interest) that go through the typical cell culture and recovery process. This technique provides a practical solution for the critical issue of product contamination in the HCP preparation. Alternatives to this approach are to use downstream steps in the process to enrich HCPs specific for a given process (''process specific''), however this has the downside of being overly specific and (1) if the process is changed even subtly, the HCP profile may also change, making the detectability less relevant and (2) if a process failure results in an unexpected HCP carrying through, the assay is underpowered to detect and measure it.

Ultimately, the HCP preparation is used as a standard for testing to assure accurate measurements of residual HCPs in final drug substance samples. Therefore, a main objective in developing anti-HCP antibodies is to obtain a reagent capable of detecting a broad range of HCPs and with sensitivity at parts per million levels in the drug substance. If one assumes that a preparation of HCP immunogens that are derived from a null cell line contains 10 ppm biologic drug (contamination), and we also assume that the drug elicits a typical immune reaction from the animals used to raise the anti-HCP antibodies, we would expect to have anti-product antibodies at 10 ppm in the anti-HCP antibody preparation. According to the dissociation equation $K_d = [A][B]/[AB]$, in a drug substance preparation with HCPs at 10 ppm level, the complex formed between the biologic drug and its corresponding antibody [10 (anti-Protein product antibodies)][999,990 (Protein product molecules)] is equal to the number of complexes formed by anti-HCP antibodies and HCPs in the drug substance [999,990 (anti-HCP antibodies)][10 (HCP molecules)]. Since the quantitation of HCP with a sandwich ELISA does not distinguish between these two types of complexes, there may be interference from the drug that manifests as cross-reactivity. Therefore, lower levels of HCP in the final drug substance could result in even greater interference, perhaps one explanation for the lack of dilutional linearity that is known to occur in some programs. It is important to use a null cell line and pristine equipment (free of residual recombinant protein drug) when preparing HCP preparations.

As part of assay development, recovery of HCPs spiked into drug substance and process samples is a common practice and a valuable tool when developing quality control assays such as Western blots and ELISAs. If the goal of the

immunoassay is to detect 10 ppm or less of HCPs in the drug substance without significant interference, a spike of 2 ppm or less of drug substance should be used as positive control in a Western blot to confirm no product contamination higher than 2 ppm (Fig. 3). Currently, a large proportion of marketed biologics are monoclonal antibodies. Thus, it is relatively easy to obtain anti-human IgG antibodies from commercial source for this type of Western blot analysis. With the spiking format, if the prepared immunogen has a biologic product signal higher than the spiked sample, it suggests that there is possible contamination from the biologic product and the immunogen preparation process needs to be repeated with caution. Also, the amount of HCPs prepared for use as a standard reagent for the validated assay should be adequate to support the full development of the assay and analytical support after market approval, preferentially covering the life time of the drug. Preparing the immunogen carefully is essential since it will also be used as a raw material to standardize the quantitation antibodies. Many different approaches have been employed to raise antibodies to HCP antigens (Briggs and Panfili, 1991; Eaton, 1995; Thalhamer and Freund, 1984). Rabbits or goats are most common and some prefer to use more than one species of animals to increase the diversity of the antibody population with the aim of obtaining better coverage. It usually takes several immunizations to reach a maximum immunological response; the process could take 60–90 days to complete depending on the frequency of immunization. When the titer reaches the maximum, Western blot will be used to test the coverage of HCPs from antibodies in different animals and the antiserum from different animals are pooled to develop the ELISA assay and Western blot method. Multiple animals can also provide an additional diversity of responses to maximize antibody coverage of HCPs and for this reason rabbits are sometimes preferred over goats. Although in theory this diversity is improvement, it should be noted that it does not necessarily translate to better coverage or higher sensitivity in the immunoassay subsequently developed. The reason for this is that in the most common format of immunoassay, the ELISA, the assay is most likely performed in a 96 or 384 well microplate. For the detection of process-related impurities such as HCPs, which are usually present in the parts per million levels, a sandwich ELISA is often used (Crowther, 2001). In this format, the extremely low levels of HCPs are first enriched by the binding antibody coated on the microplate. The enriched HCPs are further recognized with a reporting antibody, which will amplify the HCP signal with an enzyme or fluorescence tag. In this format, there is a limitation of the amount of binding antibodies in the plate (usually <1 μg/well). Therefore, the more diverse the antibody preparation, the fewer the amount from each anti-HCP species will be present. If the binding antibody is a limiting factor in the assay (a very likely scenario), the diversity of the antibodies will not translate into better coverage or higher sensitivity.

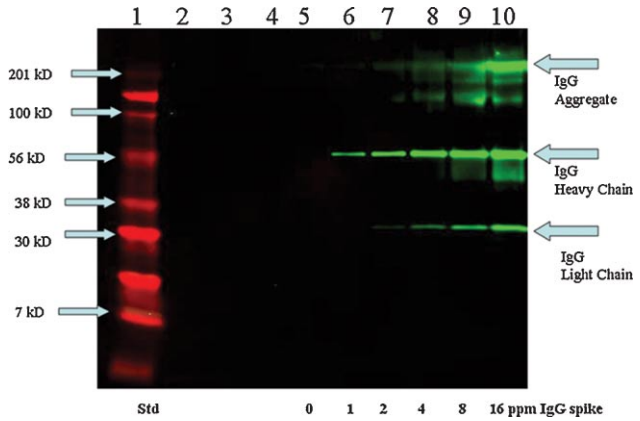

Figure 3. Western blot analysis of IgG spike in HCP immunogen preparation from a null cell culture medium **Lane** 1, molecular standards; **lane** 2–10, HCP immunogen at 10 μg/lane. An internal IgG_2 was spiked at the indicated concentrations to confirm the HCP preparation was free from IgG contamination. 10–20% gradient SDS–PAGE was used for the protein separation. Donkey anti-human IgG-IRDye-800 nm conjugate was used at 1:10,000 for IgG detection. Image was obtained by the Odyssey system from Li-Cor.

ELISA continues to be the most commonly employed and practically useful application for HCP testing, largely due to the relative ease and good precision of the technique and also that it is quantitative and provides numerical results that are amenable for setting control limits and specifications. It is important to keep in mind, however, that there are limitations as well. Before going into these, a consideration of the ELISA development process itself will be discussed. As mentioned above, as the first step, antibodies are raised against HCPs obtained from null cells. Since the immunogenicity of each HCP depends on the primary structure, conformation state and abundance of the protein, there are several possible outcomes in the reagent antibody generation step. Firstly, a group of HCPs may (ideally) generate at least two high quality antibodies that recognize different epitopes on the HCP. In this case, a sandwich ELISA will detect the corresponding HCPs. In the second outcome, two specific antibodies are generated, but the corresponding epitopes on the HCP are too close to allow a successful sandwich ELISA detection (detection antibody is sterically hindered and cannot bind). In the third outcome, there is only one good antibody being generated from the HCP, and obviously sandwich ELISA will not detect the corresponding HCPs (Fig. 4). The forth possible outcome is that no antibodies will be generated because of the weak immunogenicity of the HCPs or HCPs exist at very low level in the immunogen preparation. Under these scenarios, only the first one will reliably detect the HCP. However, most consider this risk low since the immunogenic nature of HCPs and the diversity afforded by polyclonal antibody responses (as well as the procedures mentioned above) would reasonably be expected to be the normal outcome. In contrast, only one specific antibody is required for HCP detection in Western blots. This value is

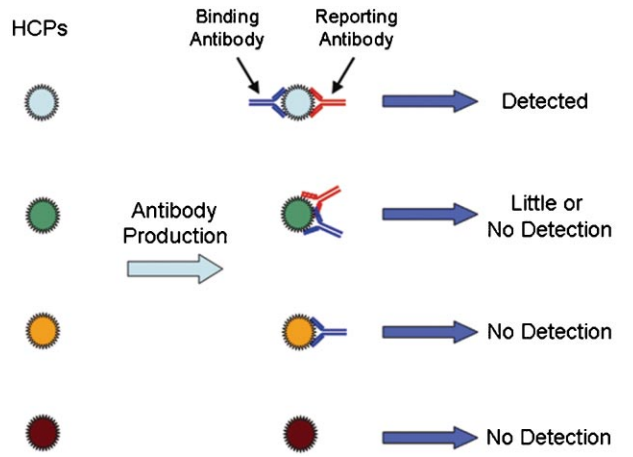

Figure 4. Possible outcomes of immune responses toward Host Cell Proteins and their detection.

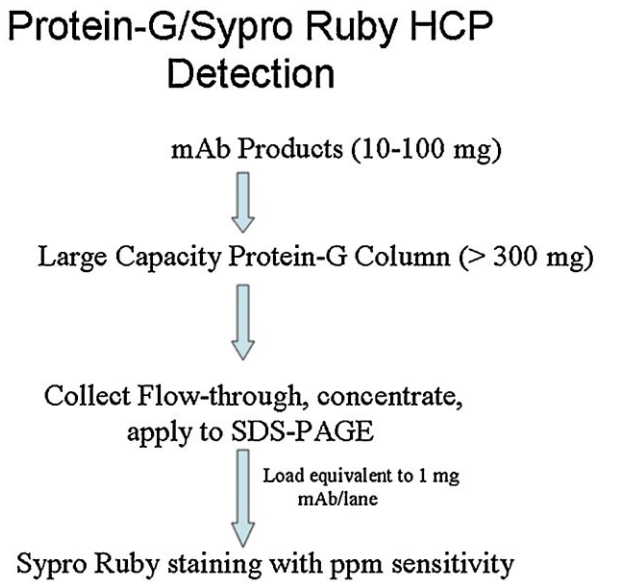

Figure 5. Flow chart of the Protein G and Sypro Ruby detection of Host Cell Proteins.

offset by another consideration that the denatured HCPs after SDS–PAGE are not in the natural, native state and may be missed. These considerations are why both procedures are recommended, particularly in the early stages of development to assure that orthogonal methods are in place and the best assay is chosen.

Because the nature of HCP testing is complex, and conditional upon many factors, no one procedure or modality is recommended by regulators and sponsors will look at a number of options. Novel approaches have been tried, though publications in refereed journals are rare, and industry continues to look for more standard approaches. One method that we are investigating has been evaluated with monoclonal antibodies. This method has a pre-treatment step with Protein G resin to capture a majority of the therapeutic protein and subsequent analysis of the unbound, enriched HCP fraction using Sypro Ruby detection (Fig. 5). Protein G is used instead of Protein A since the latter has already been used in bioprocessing for capture, and our aim is to monitor HCPs that might bind to Protein A at low levels non-specifically during purification. After concentration of HCPs, separation by SDS–PAGE and blotting, the membrane is analyzed after probing with antibodies to human IgG. A separate SDS–PAGE gel is stained for total protein using Sypro Ruby (Fig. 5). The proteins that are not recognized with polyclonal anti-human IgGs but detected by Sypro Ruby staining are probably HCPs, now available for identification by mass spectrometry if necessary. The sensitivity of this method and robustness are under evaluation.

Generic Versus Process-Specific HCP Assays

Currently, there are several commercial HCP detection kits available for industrial use that biotechnology companies continue to evaluate and to compare with their own, as well as develop their own customized kits (Champion et al., 2001; Eaton, 1995; Hoffman, 2000). For commercial ELISAs, the main advantage is that no time and resources are necessary for antibody generation, method development, and quality control. A disadvantage of a commercial HCP ELISA kits is that the generic nature of the standards and antibodies may result in an assay less specific than any individual program or internal program. An example is provided in Figure 6 and Table I where the measurement of HCP levels with commercial reagents is contrasted with those from a customized version developed specifically for the process and the expression system. A very sensitive Western blot was developed to illustrate the coverage, as was an ELISA using the same antibodies prepared and conjugated for an immunoassay. It can be seen that the custom reagents "see" different bands compared to the commerical reagents, and that the ELISA results are more sensitive (In the overall process, commercial reagents see fewer bands and measure lower amounts than the customized version). This outcome is one example; in other cases the differences were not significant (data not shown). It is obvious from Figure 6 that much greater value was obtained from the customized (internal) assay and process decision-making is enhanced. Practically, other disadvantages of commercial reagents include higher cost and, perhaps most significantly, a lack of control of reagents.

Customized assays have a theoretical advantage in specificity for the particular bioprocess used for production (see Table I and Figure 6 for one case), although a thorough treatment of this subject has not been published. In any case,

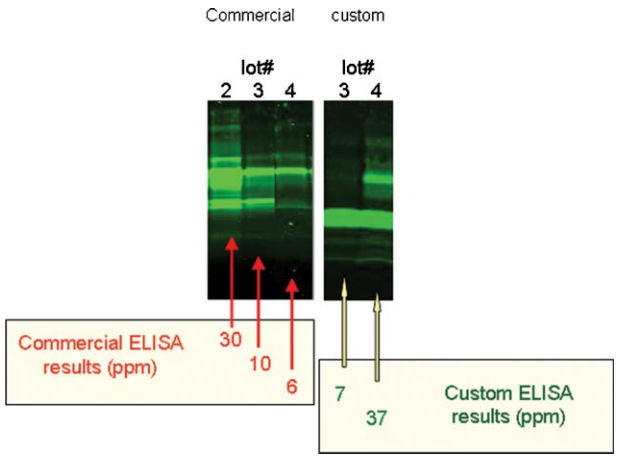

Figure 6. Western blot comparison of generic and process-specific anti-HCP antibodies in the detection of Host Cell Proteins from two bioprocesses. Drug substance from an old process was loaded at 50 μg/lane in a SDS–PAGE. Anti-HCP antibodies from both commercial source and in-house (process-specific) were diluted at 1:2,000 (1 mg/mL IgG). Secondary antibodies against Goat IgG (for commercial antibody) or against Rabbit (for process-specific antibody) with IRDye 800 nm conjugate were diluted 1:10,000 for incubation. The image acquisition is the same as in Figure 2.

empirical data is best in comparing the available reagents for specificity. As mentioned above, in many cases, the more relevant HCP population in the custom preparation is more sensitive and has better coverage (for this reason the risk of "detectability" is less in our example in Fig. 1). Another disadvantage however, is the time and labor required to develop a process-specific immunoassay. It can take 12–18 months from the generation of the null cell isolate, immunizations, to the validation of the ELISA assay and is therefore a significant commitment of time and resources. A risk assessment, as previously discussed, is a way to mitigate risk and choose the best timing for development of a custom assay.

Table I. Comparison of host cell proteins from commercial and customized (company program specific) ELISA in multiple batches of drug substance from a mid phase manufacturing campaign (*E. coli* process).

Sample name	Commercial HCP ELISA (ng/mg)	Customized HCP ELISA (ng/mg)
Batch 1	102	33
Batch 2	30	38
Batch 3	10	7
Batch 4	6	37
Batch 5	5	31
Batch 6	7	20
Batch 7	4	40
Batch 8	4	39
Batch 9	3	15
Batch 10	2	48
Batch 11	2	30

Platform-Based Approach for HCP Analysis

Another approach to overcome the major commitment of resources and time for HCP assay development is to apply a platform approach with similar strains and isolation procedures that can be applied across programs (Krawitz et al., 2006). The platform-based approach for HCP detection and characterization makes sense for a number of reasons. First, if a large number of drug candidates from the same expression system exist in a portfolio, availability of efficient and common methods for HCP analysis enable precedent, consistency and uniform support of process development for all of them. In this approach, the cell lines used for multiple drug candidate production are derived from a common starting cell line, and the protein purification process also follows a similar pattern. Because of the similarity of the cell lines, the proteome (total protein composition and relative abundance) of the HCP will be very similar (internal studies). A number of studies comparing the HCP proteomes from different production cell lines using the same platform have been reported. These studies showed that the HCP proteomes are very similar among the cell lines evaluated, providing the basis for the platform-based approach for HCP evaluation (Champion et al., 2001; Krawitz et al., 2006; Smales et al., 2004). The second more practical reason, to take the platform approach is the high attrition rate of early drug candidates. It is known that the majority of early drug candidates will fail during the development stage. Under these circumstances, it will be very costly to develop a process-specific HCP program for each biologic molecule in the pipeline because the high labor requirement and long timelines involved for each separate program.

For platform-based HCP analysis to be successful for a new drug candidate, analysis of the HCP proteome from the process is compared to that for the parent cell line. The current number of mammalian cell lines used for biologics production are relatively small, the most commonly employed being CHO and NS0 cells. Similarly *E. coli* and yeast are the two most common microbial expression systems. Therefore, with the platform-based approach, a limited number of HCP assays need to be developed. However, as mentioned previously, care must be taken when using the platform based approach for HCP analysis because the HCP patterns could potentially change with changes in both the biologic molecule of interest and the bioprocess.

Application of Proteomics, Genomics and Bioinformatics in HCP Identification and Evaluation

Advancements in the field of systems biology have changed how biology research is conducted (Weston and Hood, 2004). New technologies now available in DNA sequencing, high throughput protein identification with mass spectrometry and the development of efficient computer based

sequence search algorithms has enabled rapid developments in the field. It is expected that systems biology will have major impacts in biological research and drug development (Weston and Hood, 2004). Proteomics, genomics and bioinformatics are some of the disciplines in systems biology that find their applications in biologics development. In this section, the review of these emerging fields is confined to the understanding of HCPs and how these disciplines are used for their identification and evaluation.

Proteomics Application

Proteomics is the analysis of the entire protein complement expressed by a genome or a cell or tissue type (Wilkins et al., 1996). The major advancements in this field include. (1) The development of high throughput capability in mass spectrometry for protein identification and characterization. (2) The availability of whole genome databases from some of the common organisms used in biologics expression (such as *E. coli*, *Saccharomyces cerevisiae*, mouse, rat, and humans; Blattner et al., 1997; Goffeau 1997; Lander et al., 2001; Venter et al., 2001; Waterston et al., 2002). (3) Improved chromatographic fractionation for sample preparation. (4) Reproducible two-dimensional gel electrophoresis and more sensitive protein fluorescence staining.

Many studies have been carried out in the application of proteomics for HCP analysis (Dyk et al., 2003; Hayduk et al., 2004; Krawitz et al., 2006; Smales et al., 2004). Krawitz et al. (2006) demonstrated using 2D gels that it is possible to establish the comparability of HCPs from different biologics expressed in the same cell line, providing the basis for the platform-based HCP approach. We also use proteomics technologies to compare HCP patterns for multiple projects as well as identify specific HCPs in support of process development. For example, proteomics was used to identify two HCPs that persisted in a recombinant therapeutic protein purification process. Two-dimensional gel Western blot was used to locate the HCPs in the sample, and total protein staining from upstream process samples separated in a 2D gel was used to isolate the corresponding HCPs; this was followed by protein identification with MS/MS (Hunter et al., 2009; Fig. 7).

Besides the application in HCP identification and platform-based HCP program support, several additional applications can be found using the proteomics approach. One of them is the cell culture selection in the preparation of immunogens for the generation of HCP antibodies. Early in biologics process development, multiple cell lines, and culturing conditions are evaluated in order to select a final cell line. At this stage, it is also advisable to evaluate the HCP proteome of the different cell lines and culture conditions in order to develop a better understanding of the relevant immunogens for the HCP program and gain early insight into any difficulties that may be encountered or changes that may be necessary to platform HCP analysis methods.

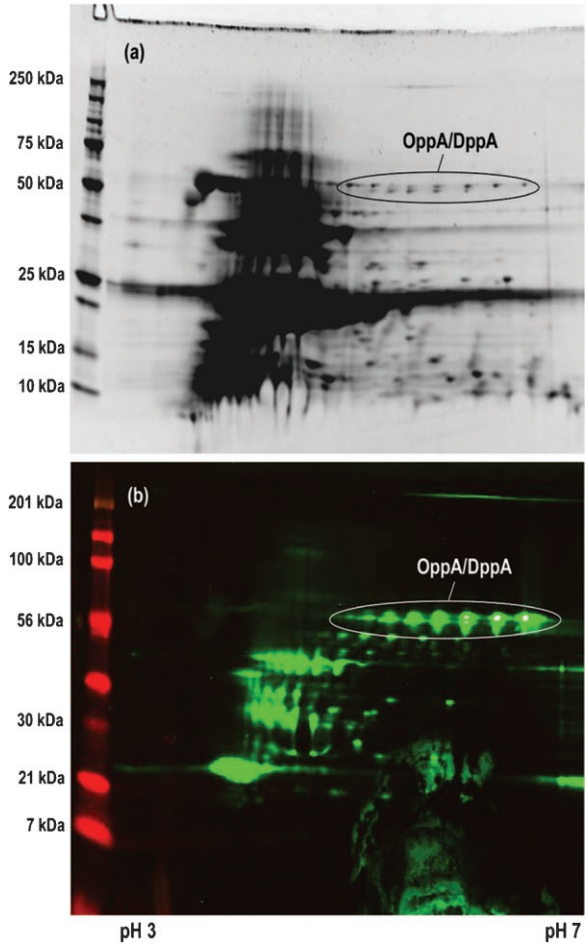

Figure 7. Proteomic approach for Host Cell Protein identification and evaluation. **a**: upstream sample was separated by 2D gel and stained by Sypro Ruby to obtain high level of HCP for MS identification. **b**: same sample was used in 2D Western blot to locate the persistent HCPs in the purification process. Four hundred microgram of total protein was loaded in each 2D gel, the two major HCPs identified are OppA and DppA, two periplasmic proteins found in *E. coli*. Reprinted from Hunter et al. (2009) with permission of John Wiley and Sons, Inc.

Genomics Application for HCP Evaluation

Genomics is the study of genes and their function. It aims to understand the structure of the genome, including the mapping of genes and sequencing of DNA (McKusick and Ruddle, 1987). A dramatic advancement in genomics research has been made in the past decade with the development in high throughput DNA sequencing. The impact of genomics in science and society has been recently reviewed (Weston and Hood, 2004). For its application in HCP identification and characterization, the most important development is the complete genomic sequencing of a number of organisms including man, mouse, rat, *Saccharomyces cerevisiae*, and *E. coli*. Those genome databases make it possible to identify HCPs found during the manufacture of biologics.

Another application from the genomics field is the further understanding of the HCP populations during the bioprocess development. Take for example, the expression of a therapeutic protein in the periplasm of *E. coli*. With the availability of the *E. coli* genome and proteomic information of periplasmic proteins, it is possible to display electronically the populations of HCPs in the periplasmic space. Those sharing a similar pI and molecular mass to the recombinant protein may be anticipated to pose purification challenges. If the HCPs prove difficult to remove with an optimized purification process, they can be identified with the previously described proteomics approach and information from genomics study of the cell line or organism will determine whether those HCPs can be knocked-out without affecting the viability or productivity of the cell.

Bioinformatics Application for HCP Identification and Risk Assessment

Bioinformatics is the field of science in which biology, computer science, and information technology come together There are three main sub-disciplines within bioinformatics: the development of new algorithms and statistics with which to assess relationships among members of large data sets; the analysis, and interpretation of various types of data including nucleotide and amino acid sequences, protein domains, and protein structures; and the development and implementation of tools that enable efficient access and management of different types of information (The National Center for Biotechnology Information, 2001). The creation and development of the bioinformatics field is closely associated with the rapid progress made in both proteomics and genomics. Bioinformatics has become an indispensable tool for biological research, drug discovery, and now HCP analysis.

One very useful way to apply bioinformatics in biologics development, especially in HCP identification and evaluation, is accessing the vast amount of information available on the internet. It is beyond the scope of this review to give a more thorough review, but three examples of bioinformatics portals and their application in HCP evaluation will be discussed. The first portal is the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). This site contains updated public information on DNA sequences and protein amino acid sequences. Various bioinformatics tools are available to search and analyze the gene or protein of interest. For example, if HCPs were identified from a bioprocess, their functions can be investigated. If the HCPs are from mammalian cell lines such as CHO or NS0, an evaluation can be done to determine if any homologous proteins from the human genome exist (and therefore the physiological impact estimated). A Blast (Basic Local Alignment Search Tool) search in the human genome will help to answer these important questions. The second portal is the Expert Protein Analysis System (http://us.expasy.org), this site contains the most comprehensive and updated tools for protein analysis. Among the numerous applications, a few examples are in order. One is called Tagldent. This algorithm allows the scientist to find the composition of HCPs from a defined organism that is similar in both isoelectric point and molecular weight. This information (number and distribution of related HCPs) could be used in the design of specific purification strategy for the protein of interest, especially for non-monoclonal antibody proteins. Another tool is the CLUSTALW program for protein sequence alignment. For example, if HCPs are identified from mammalian cells such as CHO or NS0, to find out the possibility of a functional interference from those HCPs, a protein similarity search and sequence alignment from the human genome can be performed. This will help to assess the relative risk of functional interference from HCPs identified. The third portal useful for HCP evaluation is a web site for protein immunogenicity scan (www.syfpeith.de). This is especially useful for HCPs identified from bacteria and yeast because they are evolutionarily more divergent from mammalian cells. Even HCPs from the mouse cell line could also pose an immunogenicity risk or act as adjuvant to prime the human immune system for a strong reaction (Rammensee et al., 1999). It is evident that the advancement in systems biology, especially in proteomics, genomics, and bioinformatics have greatly increased our understanding and analytical capability in the area of HCP identification and evaluation. The integration of these aforementioned fields in HCP analysis will play an important role in the development of bioprocesses for safe and efficacious biologics.

Impacts of Host Cell Protein Analysis in Bioprocess Development and Validation

The clearance of HCPs is a major consideration during bioprocess development, and levels in the final drug substance may be a deciding factor in whether the batch is suitable for clinical use. It is therefore not surprising that a large number of reports on process development have chosen HCP clearance as one of the benchmarks to demonstrate a robust and well-controlled bioprocess (Follman and Fahrner, 2004; Rathore et al., 2003; Shukla et al., 2007, 2008; Venkiteshwaran et al., 2007). Furthermore, much of our knowledge regarding HCPs in biologics was obtained through process development studies. Here we provide some examples of how HCP analysis plays a role in bioprocess development and validation.

In a recent study, Shukla et al. (2007) has demonstrated that the level of HCPs in a monoclonal antibody purification process depends on the antibody itself. Human IgGs are highly conserved with most differences in the complementarity determining regions (CDRs) being responsible for antigen recognition (Janeway et al., 2005). When antibodies are derived from a single subclass such as IgG_2, then the homology is even higher. However, when compared following the major purification step, Protein A affinity

chromatography, there is a surprisingly wide range of HCP levels (Shukla et al., 2007). These findings show that the (relatively) minor structural differences between otherwise biochemically similar (conserved sequence) IgG's can have a significant impact on HCP clearance. A related observation by Tobler et al. (2006) was made using three samples analyzed after Protein A chromatography. The first was antibody-free culture medium, the second was the same culture medium spiked with a monoclonal antibody. The HCP clearance factors after Protein A purification were compared with a third sample where the same mAb obtained from a typical cell culture medium production sample was applied to the Protein A column. It was found that the HCP clearance factor in the mAb-spiked samples was significantly lower than the mAb-free culture but very close to the typical mAb-containing sample. This result indicated that sub-populations of HCPs can co-purify with the mAb of interest, Shukla and Hinckley (2008) also reported similar findings recently.

Besides conventional chromatography, HCP clearance has been the basis for development of other novel separation techniques in biologics purification processes. To cite a few examples, HCP clearance was applied to justify a membrane-based process (Lebreton et al., 2008; Phillips et al., 2005), to demonstrate the utility of new organic modifiers for reversed phase chromatography (Hunter et al., 2008), and for high throughput screening of chromatographic separations (Kelley et al., 2008). Clearance of HCPs is commonly included as a critical process parameter during process validation. These studies are conducted by simply measuring clearance of HCPs from sample streams during process validation and comparing overall clearance. Another approach is to use a scaled-down model and apply a predetermined load of HCPs to the each process step (e.g., chromatography) used in a purification process. This type of study is intended to demonstrate that even if HCP clearance in a particular column is lower than expected, the HCP removing capabilities from the other purification steps can compensate, and produce biologics with acceptable HCP levels (Shukla et al., 2008). This latter approach may be useful but careful controls are necessary to assure the stability of the HCP preparations, the suitability of the scaled model, and the usage of appropriately represented HCPs from the actual process.

Conclusions and Future Prospects

HCP analysis and control produce an interesting conundrum for our industry insomuch as they represent the necessary machinery for living cells to express the potentially life-saving therapies that the recombinant proteins are hoped to provide. Much debate has been extended as to the significance of the risk they present. As a collection of a nearly infinite variety of structurally complex impurities, the testing strategies, and interpretation seem closer to art than science. However, it is exactly this ambiguity that demands the most robust and thorough scientific thinking that our industry can muster. Otherwise the risks imposed by HCPs are inadequately approached.

For these reasons, the detection and evaluation of HCPs is one of the important parameters in protein-based biologics development. Recent advances in the understanding of the biology and immunology associated with HCPs has helped advance the development of novel strategies for their detection and risk assessment. One size certainly does not fit all when it comes to HCP evaluation. Long time frames for antibody development are an imperative to beginning HCP evaluation early in the development cycle or risk problems and a lack of process robustness. It is important to understand the regulatory expectations regarding HCPs, and to have a solid scientific HCP strategy in place as the drug candidate advances through clinical trials. There is evidence that HCPs at sufficiently high levels will elicit an immune response, and possibly result in clinically relevant adverse events. The strategy for setting HCP specifications for any biologic depends on the capability of the purification technology and a thorough evaluation of the risk. Given the high cost of bringing a drug to market, it is imperative to use the best tools available to give a sound scientific evaluation of the nature and level of HCP impurities and minimize the potential for adverse events from HCPs.

Better understanding of HCPs continues to be a fertile field and is still evolving. New technologies are being developed and are adding to our capabilities in this area. The future challenges include the development of analytical methods that can detect all or the majority of the HCPs in the biologic to provide critical input to bioprocess development from HCP analysis, and development of new testing systems to better predict the potential immunogenicity risks posed by the presence of HCPs in biologics drug products. Rapid developments in genomics, proteomics, and bioinformatics, and other fields continues to advance our knowledge and capability for HCP analysis in biologics development.

We thank the following colleagues who contributed to many aspects of Pfizer's Host Cell Protein platform and method development: Theodore J. Kocot, Thomas Schomogy, Sa V. Ho, John T. Herberg, Mark E. Gustafson, Michael J. Dupuis, Joseph P. Martin, Paul Mensah, and Scott I. Allen.

References

Anderson NL, Anderson NG. 2002. The human plasma proteome: History, character, and diagnostic prospects. Mol Cell Proteomics 1:845–867.

Anicetti VR, Fehskens EF, Reed BR, Chen AB, Moore P, Geier MD, Jones AJS. 1986. Immunoassay for the detection of E. coli proteins in recombinant DNA derived human growth hormone. J Immunol Methods 91:213–224.

Belov ME, Anderson GA, Angell NH, Shen Y, Tolic N, Udseth HR, Smith RD. 2001. Dynamic range expansion applied to mass spectrometry based on data-dependent selective ion ejection in capillary liquid chromatography fourier transform ion cyclotron resonance for enhanced proteome characterization. Anal Chem 73:5052–5060.

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, et al. 1997.

The complete genome sequence of Escherichia coli K-12. Science 277(5331):1453–1474.
Briggs J, Panfili PR. 1991. Quantitation of DNA and protein impurities in biopharmaceuticals. Anal Chem 63:850–859.
Champion K, Nishihara JC, Joly JC, Arnott D. 2001. Similarity of the Escherichia coli proteome upon completion of different biopharmaceutical fermentation process. Proteomics 1:1133–1148.
Champion K, Madden H, Dougherty J, Shacter E. 2005. Defining your product profile and maintaining control over it, part 2. BioProcess Int 3(8):52–57.
Crowther JR. 2001. The ELISA guidebook. Totowa, NJ: Humana Press.
Dagouassat N, Haeuw J-F, Robillard V, Damien F, Libon C, Corvaia N, Lawny F, Nguyen TN, Bonnefoy J-Y, Beck A. 2001. Development of a quantitative assay for residual host cell proteins in a recombinant subunit vaccine against human respiratory syncytial virus. J Immunol Methods 251:151–159.
Dyk DDV, Misztal DR, Wiljkins MR, Mackintosh JA, Poljak A, Varnai JC, Teber E, Walsh BJ, Gray PP. 2003. Identification of cellular changes associated with increased production of human growth hormone in a recombinant Chinese hamster overy cell line. Proteomics 3:147–156.
Eaton LC. 1995. Host cell contaminant protein assay development for recombinant biopharmaceuticols. J Chromatogr A 705:105–114.
EMEA. 1997. CPMP position statement on DNA and Host Cell Proteins (HCP) impurities, routine testing versus validation studies. London: European Medicines Agency.
FDA. 1997. Points to consider in the manufacture and testing of monoclonal antibody products for human use. www.fda.gov/CBER/gdlns/ptc_mab.pdf.
Follman DK, Fahrner RL. 2004. Factorial screening of antibody purification processes using three chromatography steps without protein A. J Chromatogr A 1024:79–85.
Gibbs RA, Weinstock GM, Metzker ML, et al. 2004. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. Nature 428:493–521.
Godovac-Zimmermann J, Brown LR. 2001. Perspectives for mass spectrometry and functional proteomics. Mass Spectrom Rev 20:1–57.
Goffeau A. 1997. The yeast genome directory. Nature 387:5.
Groote DD, Zangerle PF, Gevaert Y, Fassotte MF, Bequin Y, Noizat-Pirenne F, Pirenne J, Gathy R, Lopez M, Dehart I, et al. 1992. Direct stimulation of cytokines (IL-1β, TNF-α, IL-6, IL-2, IFN-γ, and GM-CSF) in whole blood I. Comparison with isolated PBMC stimulation. Cytokine 4(3): 239–248.
Hart RA, Rinas U, Bailey JE. 1990. Protein composition of vitreoscilla hemoglobin inclusion bodies produced in Escherichia coli. J Biol Chem 265(21):12728–12733.
Hayduk EJ, Choe LH, Lee KH. 2004. A two-dimensional electrophoresis map of Chinese hamster ovary cell proteins based on fluorescence staining. Electrophoresis 25:2545–2556.
Hoffman K. 2000. Strategies for host cell protein analysis. BioPharm 13(6):38–45.
House RV. 2001. Cytokine measurement techniques for assessing hypersensitivity. Toxicology 158:51–58.
Hunter AK, Hoeltzli SD, Johnson GV, Gustafson ME, Ho SV. 2008. Use of cyclohexanedimethanol as a nonflammable organic solvent for industrial scale reversed phase chromatography. J Chromatogr A 1202:107–110.
Hunter AK, Wang X, Suda EJ, Herberg JT, Shell RE, Thomas KE, Gustafson ME, Mozier NM, Ho SV. 2009. Separation of product associating *E. coli* host cell proteins OppA and DppA from recombinant apolipoprotein A-I_{Milano} in an industrial HIC unit operation. Biotechnol Prog. DOI: 10.1002/btpr.106.
ICH. 1999. Guidance for Industry Q6B Specifications: Test procedures and acceptance criteria for biotechnological/biological products.
Janeway CAJ, Travers P, Walport M, Shlomchik MJ. 2005. Immunobiology. New York and London: Garland Science.
Kelley BD, Switzer M, Bastek P, Kramarczyk JF, Molnar K, Yu T, Coffman J. 2008. High-throughput screening of chromatographic separations: IV. Ion-exchange. Biotechnol Bioeng 100(5):950–963.
Krawitz DC, Forrest W, Moreno GT, Kittleson J, Champion KM. 2006. Proteomic studies support the use of multi-product immunoassays to monitor host cell protein impurities. Proteomics 6:94–110.
Lander ES, Linton LM, Birren B, et al. 2001. Initial sequencing and analysis of the human genome. Nature 409:860–921.
Lebreton B, Brown A, Reis RV. 2008. Application of High-performance tangential flow filtration (HPTFF) to the purification of a human pharmaceutical antibody fragment expressed in escherichia coli. Biotechnol Bioeng 100:964–974.
McKusick VA, Ruddle FH. 1987. A new discipline, a new name, a new journal. Genomics 1:1–2.
Meager A. 2006. Measurement of cytokine by bioassays: Theory and applications. Methods 38:237–252.
Nick C. 2006. Lascombe D, Stonier PD, editors. Confronting the challenges in developing a biosimilar medicinal product, Haslemere, UK: Euromed Communications.
Ohmura T, Ohmizu A, Sumi A, Ohtani W, Uemura Y, Arimura H, Nishida M, Kohama Y, Okabe N, Mimura T, et al. 1987. Properties of recombinant Hepatitis B vaccine. Biochem Biophys Res Commun 149(3):1172–1178.
Phillips M, Cormier J, Ferrence J, Dowd C, Kiss R, Lutz H, Carter J. 2005. Performance of a membrane adsorber for trace impurity removal in biotechnology manusfacturing. J Chromatogr A 1078: 74–82.
Rammensee H-G, Bachmann J, Emmerich NPN, Bachor OA, Stevannvic S. 1999. SYFPEITHI: Database for MHC ligands and peptide motifs. Immunogenetics 50:213–219.
Rathore AS, Sobacke SE, Kocot TJ, Morgan DR, Dufield RL, Mozier NM. 2003. Analysis for residual host cell proteins and DNA in process streams of a recombinant protein product expressed in Escherichia coli cells. J Pharm Biomed Anal 32:1199–1211.
Rinas U, Bailey JE. 1992. Protein compositional analysis of inclusion bodies produced in recombinant Escherichia coli. Appl Microbiol Biotechnol 37:609–614.
Rinas U, Boone TC, Bailey JE. 1993. Characterization of inclusion bodies in recombinant Escherichia coli producing high levels of porcine somatotropin. J Biotechnol 28:313–320.
Shukla AA, Hinckley P. 2008. Host cell protein clearance during protein a chromatography: Development of an improved column wash step. Biotechnol Prog 24:1115–1121.
Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. 2007. Downstream processing of monoclonal antibodies-Application of platform approaches. J Chromatogr B 848:28–39.
Shukla AA, Jiang C, Ma J, Rubacha M, Flansburg L, Lee SS. 2008. Demonstration of Robust host cell protein clearance in biopharmaceutical downstream processes. Biotechnol Prog 24:615–622.
Smales CM, Dinnis DM, Stansfield SH, Alete D, Sage EA, Birch JR, Racher AJ, Marshall CT, James DC. 2004. Comparative proteomic analysis of GS-NS0 murine myeloma cell line with varying recombinant monoclonal antibody production rate. Biotechnol Bioeng 88(4):474–488.
Speicher DW, editor. 2008. Current protocols in protein science, Chapter 10, Section II. Electrophoretic separation of proteins. Hoboken, NJ: John Wiley and Sons. p. 10.2.1–10.5.1.
Thalhamer J, Freund J. 1984. Cascade immunization: A method of obtaining polyspecific antisera against crude fractions of antigens. J Immunol Methods 66:245–251.
Tobler SA, Noyes A, Rajewski JW, Shpritzer R, Piacenza W, Tannatt M, Coffman J, Vunnum S, Kelly B. 2006. Analysis of mAb protein A chromatograpgy peak precipitates and approaches to reduce peak turbidity. San Francisco, CA: American Chemical Society. p. 151.
Ullenhag G, Bird C, Ragnhammar P, Frodin JE, Strigard K, Osterborg A, Thorpe R, Mellstedt H, Wadhwa M. 2001. Incidence of GM-CSF antibodies in cancer patients receiving GM-CSF for immunostimulation. Clin Immunol 99(1):65–74.
Veeraragavan K. 1989. Studies on two major contaminating proteins of the cytoplasmic inclusion bodies in Escherichia coli. FEMS Microbiol Lett 61:149–152.

Venkiteshwaran A, Heider P, Matoservic S, Bogsnes A, Staby A, Sharfstein S, Belfort G. 2007. Optimized removal of soluble host cell proteins for the recovery of met-Human growth hormone inclusion bodies from Escherichia coli cell lysate using crossflow microfiltration. Biotechnol Prog 23:667–672.

Venter JC, Adams MD, et al. 2001. The sequence of the human genome. Science 291:1304–1351.

Waterston RH, Lindblad-Toh K, Birney E, Rogers J, et al. 2002. Initial sequencing and comparative analysis of the mouse genome. Nature 420:520–562.

Weston AD, Hood L. 2004. System biology, proteomics, and the future of health care: Toward predictive, preventive, and personalized medicine. J Proteome Res 3(2):179–196.

Wilkins MR, Pasquali C, Appel RD, Ou K, Golaz O, Sanchez J-C, Yan JX, Gooley AA, Hughes G. 1996. From proteins to proteomes: Large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Bio/Technology 14(1):61–65.

Zhu D, Saul AJ, Miles AP. 2005. A quantitative slot blot assay for host cell protein impurities in recombinant proteins expressed in *E. coli*. J Immunol Methods 306:40–50.