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Host cell protein testing strategy for hepatitis B antigen in Hexavalent vaccine – Towards a general testing strategy for recombinant vaccines

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ABSTRACT

Background: Recombinant proteins expressed in host cell systems may contain host cell proteins (HCP) as impurities. While there is no clear evidence of clinical adverse events attributable to HCP, HCP levels and profiles must be documented to meet regulatory requirements and to understand the consistency of the biological product and manufacturing process. We present a general strategy for HCP characterization applied to a recombinant protein antigen, Hepatitis B surface antigen (HBsAg) used in a multivalent vaccine.

Methods: Polyclonal antisera raised against HCPs in process fractions from a mock preparation of the HBsAg yeast expression host, *Hansenula polymorpha*, were used to develop a quantitative sandwich ELISA to measure HCP content in batches of purified recombinant HBsAg. Batches were also subjected to SDS-PAGE and LC-MS/MS to identify detectable proteins. Batch consistency was further assessed by SDS-PAGE/densitometry purity analysis and by the ratio of specific HBsAg content (by ELISA) to total protein.

Results: Using the quantitative HCP ELISA, the HCP content showed no discernable trend in multiple HBsAg batches manufactured over a 5-year period. All batches were ≥95% pure by SDS-PAGE/densitometry, with consistent HBsAg/total protein ratios. In addition to the expected HBsAg antigen protein, LC-MS/MS analysis of three HBsAg batches identified several yeast proteins, none of which are known to cause adverse reactions in humans.

Conclusions: Analysis of multiple HBsAg batches showed consistent HCP content and identification profiles, as well as product purity and specific antigen content, demonstrating consistent manufacturing process. Recombinant vaccines, unlike therapeutic products, are administered infrequently with only small amounts of protein injected at a time. With limited potential for adverse reactions to small quantities of HCPs in purified recombinant vaccine antigens, and considering the relevant regulatory guidelines, we conclude that once consistent manufacturing process has been demonstrated, routine HCP testing in recombinant vaccine antigens is no longer required.

1. Introduction

Recombinant proteins produced in genetically modified host cells represent a growing class of biotherapeutics as well as an increasing form of vaccine antigens. A wide variety of host cells are currently in use to produce such proteins on an industrial scale, including mammalian cells (e.g., Chinese hamster ovary cells), bacteria (e.g., *Escherichia coli*), and yeasts (e.g., *Saccharomyces cerevisiae, Hansenula polymorpha*) [1–4]. Each host cell produces its own range of cellular proteins in its natural growth cycle, unrelated to the intended recombinant protein product. These host cell proteins (HCP) represent potential impurities that must be minimized as far as possible during the purification process for the final recombinant protein product [5].

Such HCP may be co-purified and concentrated through the protein purification procedures along with the recombinant protein product. Residual HCPs have the potential to affect product quality, safety and efficacy. Theoretically, repeated exposure to high concentrations of HCP may induce clinically adverse events when the biological product is used in humans. Although case examples are rare, potential risks include toxicity and possible enzymatic activity which may have deleterious effects on the therapeutic protein itself [8]; prokaryotic cell components from yeasts may cause allergenic responses, while eukaryotic cell components could induce autoimmune responses [6,7]. Such immune responses have a potential to cause clinical effect to the

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patient. However, there is no clear evidence of clinical adverse events attributable to HCP.

The potential for qualitative and quantitative batch-to-batch variations in biological products may require routine batch testing for HCP with recommended analytical tools to satisfy the regulatory requirements and ensure product safety. Regulatory authorities have strict guidelines for the routine testing and characterization of recombinant licensed products such as monoclonal antibodies (mAbs) to ensure that HCPs are removed to below acceptable levels [9,14,15]. Alternatively, a validation approach may be appropriate, based on adequate characterization during product development and process validation to demonstrate capability of the process to consistently remove HCPs. The European Medicines Agency (EMA) have noted that while a validation approach is acceptable for residual host cell DNA, the choice of routine testing or validation approach for HCP needs to be made on a "case-by-case" basis depending on the performance of the method used to identify and quantitate the HCP impurities, the design of the validation studies and the intended use of the product (i.e. dose, treatment, duration) [5].

There is currently a lack of clarity in the guidelines for the testing and monitoring for HCPs in recombinant proteins used in vaccines, in which autoimmune reactions may be a particular concern. The steps taken to improve immune responses to vaccines through enhanced antigen presentation and adjuvantation may also enhance undesired immune reactions to HCPs. However, in prophylactic situations recombinant vaccine antigens are administered in low doses and with low frequency compared with the higher quantities of multiple or regular doses of biotherapeutic products such as coagulation factors or mAbs used in therapeutic situations. For example, the hepatitis B component administered in routine infant vaccines as three doses 4–8 weeks apart in the most common schedules consists of 10 µg of recombinant Hepatitis B surface antigen (HBsAg) adjuvanted with aluminium gel. In comparison, the recombinant Factor VIIa clotting factor is administered to hemophiliac patients in doses of 90–270 µg per kg body weight every day [10], and the mAb, alemtuzumab, is administered in escalating doses from 3 to 30 mg in adults [11]. This suggests that once a vaccine product has been well characterized for HCPs during development, a validation approach will be appropriate in routine vaccine production.

Sanofi Pasteur manufactures a vaccine containing recombinant HBsAg produced in *Hansenula polymorpha* yeast. We prepared process-specific anti-*Hansenula polymorpha* antibodies, demonstrated the consistent clearance of HCPs along the purification process, monitored the consistency of residual HCP content in 50 industrial lots of purified antigen and extensively characterized the HCPs in four consistency lots of purified antigen. Our conclusion is that once a consistent manufacturing process has been demonstrated, routine HCP testing is no longer required.

2. Methods

For the purposes of this study a "mock preparation" of cell extracts of *Hansenula polymorpha* lacking the HBsAg gene was prepared according to the manufacturing process from which fractions were used to prepare polyclonal antibodies (Abs) against HCPs. These polyclonal Abs and a full range of analytical techniques were then applied to three consistency manufacturing lots of the HBsAg to investigate the HCP composition.

2.1. Production and characterization of mock preparation

The mock preparation is representative of the process currently used to prepare HBsAg for vaccines. Fractions (F1–4) were harvested from the process at four different steps: along the purification process.

Each fraction was characterized for protein content using a modified Lowry kit (Thermo Scientific, Rockford, IL, USA) and the DNA content was determined by UV spectroscopy after phenol/chloroform extraction and alcohol precipitation. To further characterize the protein profile, samples of each fraction were denatured by addition of Lithium Dodecyl Sulfate (LDS) buffer (LDS Sample buffer 3X, Invitrogen) and 2-mercaptoethanol and heating at 95 °C for 5 min. Aliquots, equivalent to 25 µg total protein for F1, and 15 µg total protein for F2, F3 and F4 were loaded on 4–12% (w/v) SDS-PAGE (XT criterion gels, Bio-Rad) using 3-(N-morpholino)propanesulfonic acid (MOPS) as running buffer. Gels were stained using R-350 Coomassie Blue (GE Healthcare).

2.2. Polyclonal HCP antibodies - rabbit immunization and protein a antibody purification

Four groups (6 per group) of New Zealand White Rabbits (KBL) received a series of four subcutaneous injections administered at 3 week intervals, of the four different cellular fractions (F1-F4; one per group) mixed with Freund's adjuvant (complete for the first injection and incomplete Freund's adjuvant for the boost), at a dose of 150 µg protein per injection. Blood samples were collected 1 or 2 weeks after each injection, and sera were tested by Western blot and by ELISA using mock fractions as coating antigen. Then sera were pooled and immunoaffinity purified on a protein A column.

ProSep®-A gel was packed into a column and washed with phosphate buffered saline (PBS) binding buffer (0.01 M phosphate, 0.138 M NaCl, 0.0027 M KCl, pH 7.4). Rabbit antisera diluted in binding buffer were applied to the column, washed twice with ≥10 vol of binding buffer, and antibodies then eluted with 0.1 M glycine-HCl (pH 2.5) in successive collecting fractions. The immunoglobulin G (IgG) concentration in each eluted fraction was measured by UV absorbance at 280 nm; fractions with an absorbance > 0.2 AU were pooled and the solution immediately dialyzed against 0.1 M potassium phosphate buffer (pH 7.4), with final Ig concentration determined by 280 nm ($\varepsilon = 0.674$). Purity was evaluated by mini-gel electrophoresis using the PhastSystem (SDS-PAGE on PhastSystem using PhastGel 10–15%, reduced and non-reduced, and PhastGel Blue R staining; Amersham Biosciences, Buckinghamshire, UK). Protein A purified anti-HCP IgG (called later anti-HCP antibodies) were stored at about −20 °C with 0.01% (w/v) sodium azide as preservative.

2.3. Antibody characterization - Western Blot

SDS-PAGE of cell extracts using XT Bis-Tris 4–12% polyacrylamide gels was performed under denaturing and reducing conditions, with 10 µg of HBsAg included as control to ensure no reactivity between vaccine components and anti-HCP antibodies. Electrophoresis was performed in 2-(N-morpholino)ethanesulfonic acid (MES) buffer, and gels were transferred onto a nitrocellulose membrane by semi-dry transfer using the iBlot™ system (Invitrogen).

For immunoblotting, membranes were incubated in buffer containing non-fat dry milk 5% (w/v) in TBS-T (Tris Buffer Saline with Tween) for at least 1 h at room temperature (RT) to block non-specific binding sites. Membranes were then incubated (1 h at RT) with the purified immune-sera containing anti-HCP diluted in TBS-T containing non-fat dry milk 2.5% (w/v). After removing unbound primary antibody by washing in TBS-T (3 × 10 min), membranes were incubated for 1 h at RT (Room Temperature) with an anti-species IgG, alkaline phosphatase conjugate diluted at 1:50,000 in TBS-T containing non-fat dry milk 2.5% (w/v). After washing, HCP bands were detected by incubating with BCIP®/NBT for 20–30 min at RT. Western Blot HCP profile images were recorded using the VersaDoc system (Bio-Rad) fitted out with conversion screen as scanner.

2.4. ELISA

HCP content was determined using a validated quantitative sandwich ELISA using defined pools of the rabbit polyclonal immunoaffinity purified antibodies directed against the *Hansenula polymorpha* fractions.