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Sensitive methods for evaluation of antibodies for host cell protein analysis and screening of impurities in a vaccine process

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ABSTRACT

Background: Host cell proteins (HCP) should be carefully monitored in vaccine production. To achieve a reliable HCP estimation, a mixture of polyclonal antibodies (pAbs) with broad affinity would be of preference. Sensitive evaluations of the pAbs are therefore of value.
Methods: Column purification of specific HCPs with affinity to the anti-HCP pAbs was compared with Western blotting of the anti-HCP pAbs binding to filter bound total lysate. The anti-HCP pAbs were used in an HCP quantification analysis using surface plasmon resonance (SPR). Host cell derived impurities from an influenza vaccine process were analyzed using 2-D DIGE analysis.
Results: The Western blotting showed a similar HCP binding pattern of anti-HCP pAbs from immunizations using two adjuvants: CFA/IFA and AbISCO®. From the column purification of HCPs, total proteins detectable were similar for all anti-HCP pAbs; however the immune response pattern differed significantly for the anti-HCP pAbs from the AbISCO® immunization. In the SPR HCP quantification assay the standard curve ranged from 0.3 to 40 μg/ml. The advantage of SPR compared with ELISA was the decreased hands on time and that the sample number was not limiting. The 2-D DIGE showed that most of the HCPs were removed at the clarification and virus capture step.
Discussion: Column purification of HCPs with affinity to the anti-HCP pAbs increased the sensitivity of affinity analysis compared with Western blotting and opened the possibility of further analysis. The anti-HCP pAbs did not interact with proteins in the virus; simplifying analysis of process samples using SPR. 2-D DIGE analysis gave a direct study of the impurity profile with the advantage of independence from antibody performance.

1. Introduction

To minimize the risk of adverse events following vaccination, host cell protein (HCP) impurities should be carefully monitored in vaccine production. According to the European Pharmacopoeia the influenza split and whole virion vaccines should not contain more than 100 μg total protein per dose. However, this is a general consideration of HCP as a total protein measurement will include also the viral proteins. In addition to being referred to as "impurities", studies of protein composition in different viruses have revealed a number of host cell proteins, not only in the lipid envelope of the virions but also in the interior of the virus particle [1,2]. Therefore, in vaccines composed of enveloped whole virus, split or live attenuated, HCP will follow the virus throughout purification. A question is how to decide which HCP proteins detected in the purified vaccine should be considered as "virus proteins" or "impurities"?

In order to give a reliable HCP estimation, a mixture of polyclonal antibodies (pAbs) with broad affinity would be beneficial, e.g. covering a large part of the host cell proteome. In addition, it would be preferable to have anti-HCP pAbs with affinity for only HCP that is not included in the virus itself. This to have the possibility to analyze the specific HCP that could be reduced by the purification process. There are different ways to produce antibodies in terms of animal species, production of lysate with or without detergents, adjuvants used in immunization and performance of the immunizations [3]. This will in turn provide a certain degree of difference between vaccine manufacturers' antibody production and give inconsistency regarding HCP analysis of the final product at different production sites and between vaccine batches. A

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reliable and sensitive evaluation of the polyclonal mix would minimize the uncertainty of the affinity diversity of antibody mixes. The pAbs are used to set up HCP concentration assays, most commonly using ELISAs. Although, today there are many other less hands on demanding alternative analyses available, such as Gyrolab™ [4], Acoustic Membrane MicroParticle (AMMP) technology [5] and proximity ligation assay with readout via real-time PCR or DNA sequencing (ProteinSeq) [6,7].

Here we show an evaluation of three different anti-HCP pAbs for Vero cells, both for the antibody binding to the HCP and the specific HCPs as such (Fig. 1). Two of the antibodies were produced in rabbits by immunization using different adjuvants: CFA/IFA and AbISCO®. They were then compared with a commercially available pAb mix. AbISCO® is a saponin based adjuvant that has been shown to be non-toxic and induce broad antibody responses including several immunoglobulin classes and subclasses, as well as potent cytotoxic T cell responses [8,9].

The specific HCP-antibodies were used in development of an HCP quantification analysis using surface plasmon resonance (SPR) with Vero cell lysate as reference (Fig. 2). SPR biosensors monitor the interactions of a molecule on the surface of a chip and make it possible to measure the binding capacity and affinity of antibodies to antigens in real-time as well as the concentration of specific molecules in a sample [10].

We also show a screening of host cell derived impurities through an influenza vaccine process using 2-D DIGE analysis, analyzing protein pattern obtained for different routes in process development (Fig. 3). 2-D DIGE implies CyDye™ labeling of all proteins prior to electrophoresis, enabling efficient protein detection as well as quantification possibilities [11,12].

2. Materials and methods

2.1. Immunization

Vero cells were lyzed by sonication in PBS pH 7.4 (Lonza) and centrifuged 30 min 3500 × *g*. The total protein concentration of the lysate was ~10 mg/ml (gel electrophoresis shown in Fig. 1A). Rabbits were immunized with Vero cell lysate mixed with CFA/IFA (Sigma) and AbISCO® (Isconova AB) respectively. The first bleedings were used to reduce protein levels in the lysate giving a high immediate response. This was made by coupling the sera on NHS-activated HP columns according to manufacturer's instruction (GE Healthcare) and let vero cell lysate passed through the column and was further immunized.

2.2. Purification of specific polyclonal antibodies

Vero cell lysate was coupled on NHS-activated HP columns. The rabbit sera (CFA/IFA and AbISCO®) were passed through the columns with PBS pH 7.4 as running buffer and the specific anti-HCP antibodies were eluted with 0.5 M NaCl (Merck), 0.2 M NaAc pH 2.5 (Merck).

2.3. Purification of specific HCP

The purified specific anti-HCP antibodies (CFA/IFA and AbISCO®) and commercial anti-HCP antibodies (Cygnus Technologies) were coupled on NHS-activated HP columns. Vero cell lysate was loaded on the columns and specific HCP was eluted with 0.5 M NaCl, 0.2 M NaAc pH 2.5. The purified specific HCPs were concentrated with Vivaspin 500 (GE Healthcare), labeled with CyDye™ DIGE Fluor minimal dyes (GE Healthcare) and run on SDS-PAGE (4–12% Gradient Bis-Tris gels, Life Technologies). All gels were scanned for CyDye™ in Typhoon™ FLA 9000 (GE Healthcare).

2.4. Western blotting

Vero cell lysate was separated on SDS-PAGE and blotted to nitrocellulose filters (GE Healthcare). The purified specific anti-HCP antibodies (CFA/IFA and AbISCO®) were buffer exchanged on NAP columns (GE Healthcare) to PBS pH 7.4 and incubated with the filters. Detection of protein bands was made by using Cy^3-labeled anti-rabbit antibody (GE Healthcare) and filters were scanned for CyDye™ in Typhoon™ FLA 9000.

2.5. HCP quantification assay

The purified specific anti-HCP antibodies were immobilized in 10 mM Acetate pH 4.5 (GE Healthcare) on Series S sensor chip CM5 (GE Healthcare) and a standard curve using Vero cell lysate was set up using HBS-EP+ 10x (GE Healthcare) as running buffer and 20 mM NaOH (Merck) as regeneration of the surface in a Biacore™ T200 system (GE Healthcare). To increase the stability of samples HBS-EP+ with 1% bovine serum albumin (Sigma) was used as sample diluent. At least two replicates were analyzed by each sample. Bradford (BioRad) and ELISA (Cygnus Technologies) were performed according to the manufacturer's instructions.

2.6. 2D-DIGE

Samples (50 μg protein) from an influenza purification process (A/H1N1/Solomon Islands grown in Madine-Darby Canine Kidney cells) were either Cy^3 or Cy^5 labeled and run on (i) isoelectric focusing (3-11NL, GE Healthcare) and (ii) SDS-PAGE using 2-D DIGE DALTtwelve system (GE Healthcare). A mix of all samples, spiked with transferrin to facilitate matching of the gels, was labeled with Cy^2 (internal control). Two samples and internal control were run together in each 2-D gel. All gels were scanned for CyDye™ in Typhoon™ FLA 9000 and evaluated in ImageQuant™ TL and DeCyder™ 2D 7.2.

3. Results

3.1. Anti-HCP antibody evaluation

The purified anti-HCP antibodies were analyzed by Western blotting against Vero cell lysate (Fig. 1A). The blotting showed a similar pattern of HCPs comparing antibodies from CFA/IFA and AbISCO® immunizations; there were at least 20 bands detected. The anti-HCP antibodies were also analyzed in Western blotting against a sucrose gradient purified influenza virus but did not show any visible binding to the influenza virus specific proteins (data not shown).

The anti-HCP antibodies were also evaluated by purifying the specific Vero cell HCPs binding to the antibodies. The three different HCPs (CFA/IFA, AbSICO® and commercial mix) were CyDye labeled and separated on gel electrophoresis (Fig. 1B). HCPs from AbISCO® and the commercial mix were labeled with Cy^5 and CFA/IFA HCP with Cy^3 and mixes of two labelings were loaded in the same well. This enabled a direct comparison of two HCPs within one lane by using an overlay picture, showing that total proteins detectable were similar between all three antibody mixes. However, the single layer picture showed that the immune response pattern differed significantly. The antibodies from the immunization with AbISCO® gave a more distinct affinity to the proteins (stronger protein bands, see single layer, Fig. 1B) compared with the CFA/IFA immunization.

A purified influenza virus was Cy^5-labeled and mixed with Cy^3-labeled HCPs from CFA/IFA immunization. The overlay picture did not show any visible interfering protein bands (Fig. 1B).