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Reporter cell lines for detection of pertussis toxin in acellular pertussis vaccines as a functional animal-free alternative to the *in vivo* histamine sensitization test

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ABSTRACT

Detoxified pertussis toxin (pertussis toxoid) is a major antigen in acellular pertussis vaccines. Testing these vaccines on the presence of residual pertussis toxin (PTx) and reversion to toxicity is performed by the regulatory required *in vivo* Histamine Sensitization test (HIST). Lack of mechanistic understanding of the HIST, technical handicaps and animal welfare concerns, have promoted the development of alternative methods. As the majority of the cellular effects of PTx depend on its ability to activate intracellular pathways involving cAMP, the *in vitro* cAMP-PTx assay was developed. Although this assay could be used to detect PTx activity, it lacked sensitivity and robustness for use in a quality control setting. In the present study, novel reporter cell lines (CHO-CRE and A10-CRE) were generated that stably express a reporter construct responsive to changes in intracellular cAMP levels. These reporter cell lines were able to detect PTx in a concentration-dependent manner when combined with fixed amounts of forskolin. The CHO-CRE cell line enabled detection of PTx in the context of a multivalent vaccine containing aP, with a sensitivity equal to the HIST. However, the sensitivity of the A10-CRE cells was insufficient for this purpose. The experiments also suggest that the CHO-CRE reporter cell line might be suitable for assessment of cellular effects of PTd reverted to PTx. The CHO-CRE reporter cell line provides a platform that meets the criteria for specificity and sensitivity and is a promising *in vitro* model with potential to replace the HIST.

1. Introduction

Acellular pertussis (aP) vaccines form the second generation of pertussis vaccines to protect children and adults against whooping cough. aP vaccines minimally contain inactivated pertussis toxin (referred to as pertussis toxoid (PTd)) and one to four other proteins (filamentous hemagglutinin, pertactin, fimbriae type 2 and/or type 3). PTd is thought to be key for vaccine-induced protective immunity [1,2], while pertussis toxin (PTx) [2,3] together with LPS [4] are considered responsible for the occasional side effects after wP vaccination and PTx for side effect of aP vaccination. For aP vaccines, regulatory authorities require monitoring for inactivation of PTx and reversion to toxicity through the murine histamine sensitization test (HIST). The HIST is based on the principle that PTx reduces the lethal dose of histamine 30–300 fold [5,6]. However, since the test lacks mechanistic understanding, suffers from standardization problems and causes severe animal suffering, there is an urgent need for an alternative test method.

Alternative methods for the HIST should take into consideration the four main steps involved in PTx-mediated toxicity, being cell binding, internalisation, translocation and enzymatic activity. PTx is an AB_5 toxin of which the B-oligomer is responsible for binding of PTx to glycoproteins on cell membranes, resulting in transport of the toxin to Golgi and the endoplasmatic reticulum, upon which the A subunit is released into the cytosol. In the cytosol, the A-subunit ADP-ribosylates the α-subunit of inhibitory G (G_i)-coupled receptors [7,8]. Consequently, the α-subunit can no longer inhibit adenylate cyclase (AC), an enzyme that converts ATP into cAMP.

A combination of a HPLC assay measuring the enzymatic activity of PTx [9] and a fetuin-binding assay is under development as an alternative to the HIST [10,11]. However, these assays do not assess the internalisation and translocation of the toxin. A second

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alternative method is based on PTx-induced clustered growth of CHO cells [12], which requires an enzymatically active A-subunit [13], but also cell binding, internalisation and translocation of the A-subunit to the cytosol. Current application of this test is limited to bulk products, since adjuvant-salts in final products have cytotoxic effects. Interestingly though, a recent study has shown that these cytotoxic effects can be overcome by the use of transwell inserts [14]. Nevertheless, quantification of PTx levels is restricted to manual reading due to lack of a quantitative read-out parameter, which might increase variability in test outcomes.

Previously, we have developed an A10 cell-based assay that determines the effect of PTx on G_i-coupled receptors and AC function by measuring intracellular cAMP levels, requiring cell binding, internalisation, translocation and intracellular enzymatic activity [15]. To improve sensitivity and reduce variability of this assay, we generated reporter cell lines carrying a cAMP-reporter construct, using the A10 cell lines and the PTx sensitive CHO cell line. For these reporter cell lines, optimal assay conditions and sensitivity for PTx alone and in the context of an aP containing multivalent vaccine were studied. The findings show that particularly CHO-CRE reporter cells can be used to assess residual PTx activity in aP vaccines in a quantitative manner.

2. Materials and methods

2.1. Cell lines and culture conditions

A10 rat vascular smooth muscle cells (ATCC, CRL-1476) and Chinese Hamster Ovary (CHO-K1) cells (ECACC, 85051005) were cultured (37°C, 5% CO_2) in DMEM with pyruvate (Gibco) and F-12 Ham's (Sigma Aldrich), respectively. Media were supplemented with 10% fetal calf serum (v/v), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.3 mg/ml L-glutamine (Gibco). Cells were passaged every 2–4 days with trypsine-EDTA.

2.2. Stable transfection of A10 and CHO cells with CRE-reporter

A10 and CHO cells were transfected with pNL(NIucP/CRE/Hygro) (Promega) using Viafect (Promega) as described by the manufacturer. The vector contains a minimal promotor and a cAMP responsive element (CRE) controlling the transcription of NanoLucP luciferase [16]. Stable transfectants were selected with 500 μg/ml hygromycin and single clones obtained by limiting dilution. Two clones of each of the resulting CHO-CRE and A10-CRE reporter cell lines, clone 10 and 17 and clone 20 and 28 respectively, were selected for further analysis.

2.3. Reporter assay

For optimisation experiments (Figs. 1–3), A10-CRE and CHO-CRE cells were cultured overnight (ON) in 96-well F-bottom plates at a concentration of 1×10^4 and 2.5×10^4 cells/well (100 μL/well), respectively. Cells were exposed to medium, PTx (BRP1; the reference preparation of the European Directorate for the Quality of Medicines & Health Care (EDQM)), vaccine or DMSO (Merck) and stimulated with norepinephrine, isoprenaline or forskolin (Sigma-Aldrich). Incubation times for PTx, vaccine and forskolin varied and are described in detail in the figures legends.

For experiments with vaccines (Figs. 4 and 6 and Supplementary Figs. S3–S6), A10-CRE and CHO-CRE cells were cultured in 24-well plates ON at 0.625×10^4 and 2×10^4 cells/well (250 μL/well), respectively. After the example of a previous collaborative study organised by EDQM (BSP114, phase 2 [14]), wells in which cells were exposed to vaccine carried inserts (0.4 μm, Pore Polycarbonate Membrane, Corning), to prevent direct contact between aluminium-salts of the vaccine and cells, thereby circumventing the cytotoxic effect of commercially available multivalent vaccine containing aP.

The DTaP-IP vaccine (diphtheria and tetanus toxoid, aP, inactivated polio, Netherlands Vaccine Institute, The Netherlands) used in this study, contains glutaraldehyde-formaldehyde inactivated PTx and complied with the HIST for absence of residual toxicity and reversion to toxicity (data not shown). Vaccine and medium were 'spiked' with the indicated concentrations of PTx for 1 h at 4°C and were either used directly or stored at 4°C or 37°C for 4 weeks to evaluate reversion to toxicity. Before exposure to the cells, the vaccines were centrifuged at 2000 rpm, the supernatant was removed and replaced by the same volume of medium (after the example of BSP114 phase 2 [14]). The pellet fraction of the vaccine was used for stimulation of the cells. After stimulation with forskolin for the periods described in the legends, plates were left at room temperature (RT) for 10 min, followed by addition of Nano-Glo luciferase substrate (Promega). Luciferase activity was measured for 0.1 s. using a Berthold Centro lb960 reader.

2.4. Fetuin binding assay to detect the fraction of PTx in the pellet and supernatant of spiked vaccines

To examine which portion of the spiked PTx bound to the adjuvant and which portion remained in the aqueous phase, both phases were tested in the carbohydrate binding assay according to the protocol of Xing *et al.* [17]. Briefly, the vaccine was spiked as described above on the day of use or were left at 4°C of 37°C for four weeks. Subsequently the vaccine was centrifuged at 2000 rpm for 10 min. The supernatant was transferred to micronic tubes. The pellet was dissolved in 650 μL freshly prepared 10% citrate buffer in physiological saline solution overnight at 37°C, followed by centrifugation at 2000 rpm for 10 min. To examine the amount of PTx present in the supernatant and pellet fraction, fetuin coated plates were used and PTx was detected using anti-PT sheep serum (NIBSC Code: 97/572; 1/10,000 dilution) and horse radish peroxidase labelled anti-sheep IgG (Sigma Aldrich Cat. No. A-3415; 1/2000 dilution).

2.5. CHO cell clustering assay

Clustering capacities of the CHO-CRE cell clone and parental CHO cells were studied by culturing the cells (2×10^4 cells/well) in a 24-well plate for 3 h, followed by exposure to indicated concentrations of PTx for 48 h. 24-well inserts were used, when cells were exposed to DTaP-IP vaccine. CHO cell clustering was visualised using an Olympos CKX41 microscope and Olympos UC30 camera.

2.6. Immunoblotting of multivalent DTaP-IP vaccine

For immunoblotting, PTx-spiked or non-spiked DTaP-IP vaccines were desorbed for 2 days using 3.4 mM Trisodium citrate dihydrate (Sigma-Aldrich). Subsequently, the samples were concentrated using Amicon Ultra 0.5 mL centrifugal filters with a 3 kDa cut-off (Millipore) and boiled in loading buffer. The resulting samples were loaded on denaturing 10% NuPage polyacrylamide gels (vaccine samples: 2–2.4 μg of protein, PTx: 1 μg of protein) and separated using MES running buffer. Gels were either stained with Coomassie or transferred to nitrocellulose membranes. Blots were incubated with a monoclonal α-S1 IgG antibody (ab37686, Abcam), goat-anti-mouse IgG antibody labelled with IR800 and scanned using the Odyssey infrared imager.