Vaccine xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Identification of biomarkers to detect residual pertussis toxin using microarray analysis of dendritic cells

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ARTICLE INFO

Article history:
Received 27 April 2013
Received in revised form 18 August 2013
Accepted 27 August 2013
Available online xxx

Keywords:
Pertussis toxin
Safety
Microarray
Dendritic cells
IL-2

ABSTRACT

In this study we aimed to identify genes that are responsive to pertussis toxin (PTx) and might eventually be used as biological markers in a testing strategy to detect residual PTx in vaccines. By microarray analysis we screened six human cell types (bronchial epithelial cell line BEAS-2B, fetal lung fibroblast cell line MRC-5, primary cardiac microvascular endothelial cells, primary pulmonary artery smooth muscle cells, hybrid cell line EA.Hy926 of umbilical vein endothelial cells and epithelial cell line A549 and immature monocyte-derived dendritic cells) for differential gene expression induced by PTx. Immature monocyte-derived dendritic cells (iMoDCs) were the only cells in which PTx induced significant differential expression of genes. Results were confirmed using different donors and further extended by showing specificity for PTx in comparison to *Escherichia coli* lipopolysaccharide (LPS) and *Bordetella pertussis* lipo-oligosaccharide (LOS). Statistical analysis indicated 6 genes, namely *IFNG*, *IL2*, *XCL1*, *CD69*, *CSF2* and *CXCL10*, as significantly upregulated by PTx which was also demonstrated at the protein level for genes encoding secreted proteins. IL-2 and IFN-γ gave the strongest response. The minimal PTx concentrations that induced production of IL-2 and IFN-γ in iMoDCs were 12.5 and 25 IU/ml, respectively. High concentrations of LPS slightly induced IFN-γ but not IL-2, while LOS and detoxified pertussis toxin did not induce production of either cytokine. In conclusion, using microarray analysis we evaluated six human cell lines/types for their responsiveness to PTx and found 6 PTx-responsive genes in iMoDCs of which *IL2* is the most promising candidate to be used as a biomarker for the detection of residual PTx.

1. Introduction

Pertussis disease (or whooping cough) is caused by infection with the gram-negative bacterium *Bordetella pertussis*. Since the course of this disease can be severe, especially in young children, immunization programs include pertussis vaccines. Pertussis toxin (PT), one of the major virulence factors produced by *B. pertussis*, is the principal antigen in all acellular pertussis vaccines in use today. PT is a typical A-B toxin, of which the enzymatic A protomer is responsible for ADP-ribosylation of the α subunit of G_i proteins while the B protomer is involved in binding and entry into the target cell [1,2]. The dose of biologically active PT (PTx) that would be required for effective immunization is toxic and therefore PTx is detoxified to produce pertussis toxoid (PTd) which is then used in the final formulation of the vaccines [3]. After the detoxification process however, there may still be low levels of PTx present, either because detoxification was not complete or because there was reversion of PTd back to PTx [4]. To ensure that any residual PTx is below safe levels, extensive safety testing for residual PTx in every new batch of vaccine is required by regulatory authorities [5–7]. To assess the safety of pertussis vaccines, two tests are specifically named in the European Pharmacopoeia, *i.e.* the *in vivo* histamine sensitization test (HIST) in mice and the *in vitro* Chinese Hamster Ovary (CHO) cell clustering assay [5]. Because the aluminum adjuvants in final vaccine formulations are toxic for CHO cells, the HIST is currently the only test that can be used for safety evaluation of final vaccine formulations. The HIST is based on the observation that mice injected with PTx are sensitized for histamine, which results in a decrease of the lethal dose of histamine [8]. The HIST is a lethal mouse test, although modifications have been proposed to reduce the lethality of this assay [9–11]. Additionally, the HIST suffers from large variations in inter-laboratory test performance caused by differences in test protocols used between testing sites [12,13]. Together, this makes *in vitro* alternative tests highly desirable. Several *in vitro* alternatives are currently under development, *i.e.* a cAMP assay in rat A10 cells [14], a fetuin binding assay [15,16] and an enzymatic HPLC method [15]. These assays reflect known

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http://dx.doi.org/10.1016/j.vaccine.2013.08.082

activities of PTx, *i.e.* ADP-ribosylation of G proteins (HPLC method), the binding capacity of the toxin (fetuin binding assay) or a combination of the two including cellular entry of the toxin (cAMP assay). However, not all effector mechanisms of PTx are completely understood, nor are all of its clinical effects [17,18]. In search for biomarkers indicative of the presence of PTx, instead of focusing on known mechanisms by which PTx induces biological effects, we decided to use microarray gene expression analysis as an alternative approach to identify all genes affected by PTx in cell types that are implicated in the biological effects of PTx *in vivo*. *B. pertussis* infection, in which PTx is seen as a prime virulence factor, is associated with marked leukocytosis, hyperinsulinemia, bronchopneumonia and refractory pulmonary hypertension [18,19]. Cell types involved in these physiological effects in which PTx is implicated are pancreatic cells (islets of Langerhans), smooth muscle cells, barrier cells, *i.e.* epithelial and endothelial cells, and a variety of immune cells including neutrophils, macrophages, dendritic cells and T cells. Aiming to include all biological effects of PTx, cell types were selected in which both ADP-ribosylation-dependent effects of PTx have been described as well as ADP-ribosylation independent effects, *i.e.* receptor-mediated signaling *via* the B-oligomer. Both barrier cells and immune cells fit these criteria [20–23]. Six cell lines or primary cell types all of human origin (human bronchial epithelial cell line BEAS-2B, human fetal lung fibroblast cell line MRC-5, primary human cardiac microvascular endothelial cells (HMVEC), primary human pulmonary artery smooth muscle cells (HPASMC), human cell line EA.Hy926 (a hybrid of umbilical vein endothelial cells and epithelial cell line A549) and human immature monocyte-derived dendritic cells (iMoDCs)) were selected and exposed to PTx. A panel of PTx-induced genes was found in iMoDCs exposed to 250 IU/ml PTx for 2 h and the data were confirmed in another, more extensive microarray experiment. Upregulated gene expression was subsequently confirmed using quantitative RT-PCR and ELISA techniques were employed to measure protein levels of four candidate markers: XCL1, CXCL10, IL-2 and IFN-γ. From these data, the cytokines IL-2 and IFN-γ proved to be the most promising biomarkers and the assay was further optimized by determining the sensitivity, specificity and limit of detection. In conclusion, we show here that by meticulously unraveling the effects of PTx on gene expression, a relevant and promising biomarker assay can be developed to detect PTx. Before this assay can be employed for the detection of residual PTx in final vaccine formulations, several hurdles have to be taken concerning sensitivity and toxicity of aluminum adjuvants. However, we feel that the approach we employed here can have great promise to detect biomarkers that can be used for the development of new (vaccine) safety tests.

2. Materials and methods

2.1. Reagents

The WHO-standard pertussis toxin JNIH-5 (PTx) was purchased from NIBSC (Potters Bar, UK). Each vial contained 10 μg PTx corresponding to 10×10^3 international units (IU) PTx which was completely resuspended in 1 ml of PBS + 25% glycerol. The European Pharmacopoeia reference endotoxin from *Escherichia coli* 0113:H10.k (LPS) was obtained from EDQM (Strasbourg, France). *B. pertussis* lipo-oligosaccharide (LOS) was a kind gift from Dr. Watanabe (Kitasato University, Tokyo, Japan). Freeze-dried PTx, LPS and LOS were dissolved in PBS with 25% glycerol, aliquoted and stored at −80 °C until use. For every experiment, new vials were thawed to avoid repeated freeze–thaw cycles. To determine specificity of observed responses, pertussis toxin (PTx-GSK) and pertussis toxoid (PTd-GSK) were kindly received from GlaxoSmithKline (Rixensart, Belgium).

2.2. Cell culture

Six human cell types were used in this study. Human bronchial epithelial cell line BEAS-2B and human fetal lung fibroblast cell line MRC-5 were purchased from ATCC (Manassas, VA, USA). Primary human cardiac microvascular endothelial cells (HMVEC) were obtained from Lonza (Basel, Switzerland). Primary human pulmonary artery smooth muscle cells (HPASMC) were purchased from CellSystems (Troisdorf, Germany). The human cell line EA.Hy926, a hybrid of umbilical vein endothelial cells and epithelial cell line A549, was a kind gift from Sanquin Blood Supply Foundation (Amsterdam, The Netherlands). Finally, immature monocyte-derived dendritic cells (iMoDCs) were cultured from blood of healthy, anonymous donors who gave informed consent (Sanquin Blood Supply Foundation, Amsterdam, The Netherlands). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by Ficoll-Paque (GE Healthcare) density gradient centrifugation and cultured in dendritic cell culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM HEPES (N-2-hydroxyethylpiperazin-N′-2-ethane sulfonic acid), 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco, Invitrogen, Carlsbad, CA, USA)). After isolation, PBMCs were seeded in culture plates. After 90 min, medium containing non-adherent cells was removed and DC culture medium supplemented with 100 ng/ml GM-CSF and 50 ng/ml IL-4 (both Immunotools, Friesoythe, Germany) was added. Cytokines were replenished on day three. On day six iMoDCs were used for incubation experiments.

2.3. Incubations for microarray experiments

Incubations were performed in 6-wells plates. Cells were incubated for 2 h with 1 ml of 250 IU/ml PTx, 1 IU/ml (=0.1 ng/ml) LPS, 0.1 ng/ml LOS or with PBS + 0.625% glycerol as a negative vehicle control. For the incubations in which the six different human cell types were exposed to vehicle or PTx, two independent experiments were performed with each experimental group consisting of four replicates. For additional experiments in which only iMoDCs were exposed to vehicle, PTx, LPS or LOS, four independent experiments were performed with each experimental group consisting of four replicates.

Cells were collected in RNA protect (Qiagen, Venlo, The Netherlands) to stabilize RNA. Total RNA was purified using the RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. The quantity of RNA in each sample was measured using a NanoDrop Spectrophotometer (NanoDrop technologies, Wilmington, DE) and RNA integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) using the RNA 6000 Nano Chip Kit (Agilent Technologies). RNA from the four replicates was pooled, resulting in two independent samples for each condition tested (vehicle and PTx) in the cell comparison experiment, and four independent samples for each condition (vehicle, PTx, LPS and LOS) in the iMoDC experiment.

2.4. Microarray experiments and data analyis

RNA was further processed for hybridization to Affymetrix HT HG-U133 + PM Array Plates at the Microarray Department of the University of Amsterdam, The Netherlands. RNA amplification, labeling and genechip hybridization, washing and scanning were carried out according to Affymetrix protocols.

Please cite this article in press as: Vaessen SFC, et al. Identification of biomarkers to detect residual pertussis toxin using microarray analysis of dendritic cells. Vaccine (2013), http://dx.doi.org/10.1016/j.vaccine.2013.08.082