Vaccine 29 (2011) 5114–5121

Contents lists available at ScienceDirect

Vaccine

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

Response of MUTZ-3 dendritic cells to the different components of the *Haemophilus influenzae* type B conjugate vaccine: Towards an *in vitro* assay for vaccine immunogenicity

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ARTICLE INFO

Article history:
Received 15 March 2011
Received in revised form 9 May 2011
Accepted 15 May 2011
Available online 30 May 2011

Keywords:
Dendritic cells
Haemophilus influenzae type B
MUTZ-3
Immunogenicity
In vitro assay

ABSTRACT

Potency testing is mandatory for vaccine registration and batch release. Due to various limitations to *in vivo* potency testing, there is need for relevant *in vitro* alternatives. These alternative tests should preferably comprise cells from the target (human) species. The whole suite of immune responses to vaccination that occur *in vivo* in humans cannot be tested *in vitro* using a single cell type. Even so, dendritic cells (DC) form an important candidate cell type since they are pivotal in inducing and orchestrating immune responses. Cell lines are preferred over *ex vivo* cells for reasons of safety, accessibility, and reproducibility. In this first feasibility study we used the human cell line MUTZ-3, because it most closely resembles *ex vivo* human DC, and compared its response to monocyte-derived DC (moDC).

Haemophilus influenzae type B (HiB) vaccine was chosen because its components exert different effects *in vivo*: while the HiB antigen, polyribosyl ribitol phosphate (PRP) fails to induce sufficient protection in children below 2 years of age, conjugation of this polysaccharide antigen to outer membrane protein (OMP) of *Neisseria meningitides*, results in sufficient protection. Effects of PRP, OMP, conjugated PRP–OMP, and adjuvanted vaccine (PedVax HiB), on cytokine production and surface marker expression were established. PRP induced no effects on cytokine production and the effect on surface marker expression was limited to a minor decrease in CD209 (DC-SIGN). In both MUTZ-3 and moDC, OMP induced the strongest response both in cytokine production and surface marker expression. Compared to OMP alone conjugated PRP–OMP generally induced a weaker response in cytokine production and surface marker expression. The effects of PedVax HiB were comparable to conjugated PRP–OMP. While moDC showed a larger dynamic range than MUTZ-3 DC, these cells also showed considerable variability between donors, with MUTZ-3 DC showing a consistent response between the replicate assays. In our view, this makes MUTZ-3 DC the cells of choice. In conclusion, our results demonstrate that the MUTZ-3 DC assay allows discrimination between compounds with different immunogenicity. The potential of this cell line as (part of) an *in vitro* immunogenicity assay should be further explored.

1. Introduction

The efficacy of vaccines is dependent on their ability to induce an immune response of the appropriate type and of sufficient vigour, in order to induce lasting immunoprotection. For the purpose of vaccine marketing authorisation and batch release, testing its immunogenic potential in a relevant model is mandatory. Currently, most vaccine efficacy testing is performed in animal models with their inherent limitations. Firstly, animal models are only partly representative for the human immune response. Secondly, such *in vivo* tests generally show a high variability. Thirdly, in line with the 3R principle such as formulated by the EU (86/609/EEC) there is a desire to replace animal models with *in vitro* alternatives, especially in case of regulatory testing.

These issues can potentially be addressed by development of *in vitro* assays using human immune cells, either primary cells or immortalized cell lines. Using cells of human origin has the advantage of a lack of interspecies differences. Cell lines are preferred over *ex vivo* cells for reasons of safety, accessibility, and reproducibility. The whole suite of immune responses to vaccination that occur *in vivo* in humans cannot be tested *in vitro* using a single cell type. Even

Abbreviations: DC, dendritic cells; HiB, *Haemophilus influenzae* type B; LPS, lipopolysaccharide; moDC, monocyte-derived DC; OMP, outer membrane protein 6f *Neisseria meningitides*; PRP, polyribosyl ribitol phosphate.

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doi:10.1016/j.vaccine.2011.05.050

so, dendritic cells (DC) form an important candidate cell type since they are pivotal in inducing and orchestrating immune responses [1]. In recent years several dendritic cell lines have been developed to study their response to immunogens and their role in the immune system [2].

The myeloid leukemia cell line MUTZ-3 is the immortalized equivalent of CD34+ haematopoietic progenitors. To develop from their progenitors MUTZ-3 cells require the same cytokines (GM-CSF, IL-4, and TNF-α) as monocyte-derived DC (moDC). They can be induced to a phenotype consistent with interstitial or Langerhans DC, i.e. acquisition of CD14 and loss of CD1a, with interstitial DC being DC-SIGN+ and Langerhans DC being Langerin+ [3,4]. Maturation resulted in increased expression of CD40, CD54, CD80, CD86, and HLA-DR. MHC class I, class II, and invariant (CD1) antigen processing and presentation is functional [3]. Comparison of various cell lines with moDC by gene profiling revealed that MUTZ-3 is the cell line that most closely resembles moDC [5]. Thus, DC derived from MUTZ-3 have several important characteristics in common with moDC, making the cell line a suitable alternative to DC obtained from blood donors. Furthermore, MUTZ-3 DC have already yielded promising results when explored as alternative model system to cord blood derived primary CD34+–DC for chemical contact allergens [6].

In our experimental set up we used the *Haemophilus influenzae* type B (HiB) vaccine, which does not require animal testing for batch release, because an HPLC assay for the polyribosyl ribitol phosphate (PRP) content has been accepted as potency test for this vaccine. However, HiB vaccine was chosen because it consists of 3 components each of which have a different contribution to the immune response. The capsular polysaccharide, PRP is the antigenic component of the vaccine to which the antibody response should be directed. However, a vaccine consisting only of PRP appeared only effective in adults and older children and failed to yield protection in children younger than 2 years, which is the group with the highest burden of disease [7,8]. Therefore, a second-generation HiB vaccine was developed, in which PRP is conjugated to an immunogenic protein [9,10]. Outer membrane protein from *Neisseria meningitides* (OMP) as immunogenic protein resulted in the most immunogenic conjugate vaccine tested in infants younger than 6 months of age [10]. In case of Liquid PedVax, similarly, PRP is conjugated to OMP. Furthermore, aluminium is present as adjuvant.

In the present study, we compared the effects of PRP, OMP, conjugated PRP–OMP and the adjuvanted vaccine on cytokine production and CD marker expression of MUTZ-3 DC and moDC.

2. Materials and methods

2.1. MUTZ-3 cell line

The human CD34+ acute myeloid leukemia cell line MUTZ-3 (DSMZ, Braunschweig, Germany) was cultured in the presence of α-Minimum Essential Medium (Gibco), supplemented with 20% heat-inactivated FBS (Hyclone Laboratories), penicillin (100 U/ml)/streptomycin (100 μg/ml) (Gibco), 2 mM L-glutamine (Gibco), 50 μM 2-mercaptoethanol (Serva) and 27.5 U/ml GM-CSF (PeproTech).

The cells were maintained in 12-well tissue culture plates (Costar) at a concentration of 1–5 × 10^5/ml. The cells were passed twice a week.

2.2. In vitro generation of MUTZ-3-derived immature interstitial DC

MUTZ-3 DC were cultured in 12-well tissue-culture plates at a concentration of 1 × 10^5/ml in the presence of GM-CSF (1000 U/ml; PeproTech), IL-4 (1000 U/ml; MACS, MiltenyiBiotec) and TNF-α (2.5 ng/ml; Strathmann Biotec) for 7 days. Fresh cytokines were added after 3 days.

2.3. In vitro generation of immature DC from human buffycoat

Buffycoats were obtained from Sanquin (Amsterdam). Peripheral blood mononuclear cells from healthy donors were isolated by density centrifugation on Lymphoprep (Axis-shield). Cells were washed, harvested, and resuspended in RPMI-1640 medium (Gibco) supplemented with penicillin (100 U/ml)/streptomycin (100 μg/ml)/L-glutamine (0.3 mg/ml) (Gibco) and 2% human serum (Harlan). The cells were seeded in culture flasks (Corning) to let them attach for 1 h at 37 °C. The flasks were rinsed several times with PBS of 37 °C, and fresh medium consisting of RPMI-1640, penicillin, streptomycin, L-glutamine, 10% heat-inactivated FBS (Hyclone), GM-CSF (500 U/ml) and IL-4 (250 U/ml) was added. Fresh cytokines were added after 3 days. The cells were harvested after 6 days.

2.4. Treatments

MUTZ-3 DC were treated in a concentration of 2 × 10^5/ml for 48 h in the presence of GM-CSF (1000 U/ml), IL-4 (1000 U/ml), and TNF-α (2.5 ng/ml). Monocyte-derived DC (moDC) obtained from buffycoat were treated in a concentration of 5 × 10^5 cells per well in 24-wells plates (Greiner) for 48 h in the presence of GM-CSF (500 U/ml) and IL-4 (250 U/ml).

The cells were treated with PRP (*H. influenzae* polyribosyl ribitol phosphate, 13 μg/ml), non-conjugated OMP (*Neisseria meningitidis* outer membrane protein complex 132–133 μg/ml; containing 7.8 μg/ml LPS), PRP-conjugated OMP (140–145 μg/ml (PRP content 13 μg/ml); containing 8.3 μg/ml LPS), Liquid PedVax HiB (10 μg OMP/ml (PRP content 0.9 μg/ml); 19 μg aluminium hydroxide pyrophosphate), LPS (lipopolysaccharide 100 ng/ml; Sigma), and TNF-α (50–75 ng/ml; Strathmann Biotec). PRP, OMP, PRP–OMP and Liquid PedVax HiB were a kind gift from Merck Sharp & Dohme. OMP is produced from *N. meningitides*. Although OMP had been purified, it still contained LPS. We therefore also tested the effects of LPS on DCs. TNF-α was used as a positive control for moDC maturation. After exposure, the supernatants were collected and kept at −80 °C.

2.5. Cytokine production

IL-6, IL-8, IL-10, IL-12p40, IL-12p70 and TNF-α were measured in the culture supernatants using the Bio-Plex Cytokine Assay (Bio-Rad). IL-23 was measured separately using an ELISA (eBioScience).

MUTZ-3 showed significant production of IL-6, IL-8, IL-12p40, and TNF-α but not of IL-10, IL-12p70, and IL-23. This observation is in agreement with the observations by Kim et al. [11] who showed absence of IL-10 and IL-12p70 production upon LPS treatment of MUTZ-3 DC (they did not measure IL-23). The fact that IL-12p40 is produced but not IL-12p70 and IL-23 suggests a deficiency in both p35 and p19 (IL-12p70 is a p40/p35 heterodimer, while IL-23 is a p40/p19 heterodimer). MoDC showed significant production of all cytokines tested.

2.6. Flow cytometric measurement of surface marker expression

After exposure, cells were harvested by adding a small volume of EDTA solution (0.5 mM in PBS) at 37 °C for 5 min, washing and resuspending in cold buffer (2 mM EDTA (Merck), 0.5% BSA (fraction V, Sigma) in PBS) at a concentration of 2–5 × 10^5 cells/ml. One hundred microlitres of this cell suspension was incubated with specific