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Antigenic fingerprinting of diphtheria toxoid adsorbed to aluminium phosphate

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ABSTRACT

The antigenicity of alum-adsorbed diphtheria toxoid (DTd) was determined in combination vaccines, containing DTd, tetanus toxoid and inactivated poliovirus. A panel of monoclonal antibodies was used, covering five epitopes, distributed over the antigen. The resulting antigenic fingerprint of DTd demonstrates consistency of adsorption at antigen level in final product combination vaccines. The antigenic quality of DTd alone, adsorbed to aluminium phosphate, was also determined and compared with pre-adsorbed toxoid (starting material as well as toxoid desorbed from aluminium phosphate). Some epitopes became less accessible after adsorption, while others became relatively better exposed. Some epitopes disappeared almost completely upon adsorption, but were re-established after desorption of the antigen. The results indicate that DTd is adsorbed to aluminium phosphate in a preferred orientation and not randomly.

1. Introduction

Aluminium phosphate and aluminium hydroxide are commonly used adjuvants in many licensed human vaccines, e.g. vaccines containing diphtheria toxoid (DTd), tetanus toxoid (TTd) and inactivated poliovirus (IPV). Despite its widespread use for many decades, the mechanism of action of aluminium salts is not yet unraveled completely. Probably, several aspects contribute to the adjuvant effect of aluminium salts. Aluminium adjuvants can stimulate the immune system by inducing endogenous danger signals [1,2]. Aluminium salts may also act as an antigen delivery system forming a depot at the injection site [3,4], although this is questioned [5]. Small antigen-coated aluminium salt particles may deliver the antigen to antigen-presenting cells more efficiently as compared to soluble antigen [6]. Furthermore, it is likely that adsorption of antigen to aluminium salts contributes positively to the adjuvant effect. There may be an optimum in the binding strength and the conformational stability of the antigen may play a role, but the degree of adsorption is considered important for induction of antibodies. Chang and co-workers obtained indications that the antibody response correlates with the degree of adsorption as it is in interstitial fluid [7]. This positive contribution of adsorption or adsorption strength on the antibody response was confirmed by others [8,9]. However, there is not always a positive effect of adsorption on the induction of antibodies [10,11]. Too strong binding may result in decreased immunogenicity [12]. Although the importance of antigen-adjuvant interaction may be antigen and species dependent, in general vaccine developers and manufacturers want to achieve high adsorption degrees and regulatory authorities demand consistent and stable adsorption process [13].

The turbidity of aluminium salts hampers quality control of the vaccine using *in vitro* methods and analytical techniques. For routine quality control, the degree of adsorption is usually established indirectly by either measuring the pre-adsorbed antigen in the supernatant or by desorbing the antigen from the alum salt. Desorption can be done by phosphate ions in case of aluminium hydroxide-adsorbed antigens [14] or by citrate buffer in case of

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aluminium phosphate-adsorbed antigens [15]. In all cases, measurements are not directly done on the adsorbed product. It has been shown that adsorbed antigen may have a different conformation as compared to the pre-adsorbed starting material [16]. For DTd it was shown that substantial conformational changes occur upon adsorption to aluminium hydroxide [17]. To assess vaccine quality, like batch-to-batch consistency and stability, there is a need to measure antigen quality in the final product, i.e. in adsorbed condition. Analysis can be done by physico-chemical analyses, e.g. fluorescence spectroscopy, circular dichroism and scanning calorimetry [16]. Although very informative, these methods can only be used for single antigens adsorbed to aluminium salts. This approach is not suitable in combination vaccines, because the methods cannot distinguish between antigens.

It is possible to quantify adsorbed antigen by direct immunoassay [18] or FACS analysis [19]. The Direct Alhydrogel Formulation Immunoassay (DAFIA) is an ELISA in which the solid phase is provided by the aluminium hydroxide. A fluorescein-conjugated anti-mouse antibody is used for detection of the antigen specific antibody bound to the aluminium hydroxide adsorbed antigen. The method was developed for a malaria antigen and showed excellent accuracy and good sensitivity, irrespective of the monoclonal antibodies that were used. As an alternative, a direct protein assay, using o-phthalaldehyde was also available for aluminium-adsorbed antigens [20]. However, this method was less sensitive and of course not antigen specific. Alternatively, a FACS-based assay has been described using polyclonal rabbit anti-sera against meningococcal proteins [19]. As the detecting antibody, goat anti-rabbit Fab fragment was used labeled with a fluorophore (Alexa Fluor 647). An additional advantage of FACS analysis is that information on the particle size of aluminium hydroxide is obtained. For routine analysis, FACS may be less suitable since most quality control labs will not have access to the equipment.

In most vaccines, aluminium salt particles are not fully saturated with antigen and there is possibility for other proteins, e.g. blocking agent, monoclonal antibodies, conjugate, to adsorb during the assay to the aluminium salt, resulting in high background signals. On the other hand, the presence of antibodies during the assay may desorb part of the antigen. An equilibrium exists between the adsorption of all proteins involved: the antigen, the antibodies used for detection as well as the blocking agent that is needed to prevent non-specific adsorption of antibodies. In this paper, we use an adapted version of the DAFIA to perform a qualitative analysis of DTd adsorbed to aluminium phosphate, using a panel of antibodies. By comparing the antigenic fingerprints of different batches of DTd-containing vaccines we investigate if product consistency can be demonstrated. In addition, we investigate whether epitope availability changes by adsorbing DTd to aluminum salts and whether epitope availability returns to pre-adsorption level after DTd desorption.

2. Materials and methods

2.1. Antigens and vaccines

DT-IPV vaccines were provided by Bilthoven Biologicals (BBio, The Netherlands). DT-IPV is a licensed product and all batches met release criteria. DT-IPV1-3 contained diphtheria toxoid bulk X (DTd-X), and DT-IPV4-6 contained bulk Y (DTd-Y). Also these pre-adsorbed diphtheria toxoids (DTd-X and DTd-Y) were obtained from BBio. Diphtheria toxin (DTx) was produced by Intravacc (The Netherlands).

The diphtheria toxin (DT79/1) is an in-house reference supplied by BBio containing 100 Lf/ml toxin. DT79/1 was used in the ELISA as a reference.

2.2. Antibodies

Anti-DTx monoclonal antibodies (mabs) Dim9, Dim24, Dim25 and Dim27 were produced at the National Institute of Public Health and the Environment (The Netherlands). All mabs recognize different epitopes located on A-fragment (Dim9 and Dim24) or B-fragment (Dim25, Dim27), as was determined by pair-wise binding of antibodies (primary antibody-antigen-second antibody) by Biosensor analysis [21]. Contrarily, Dim27 binds mainly to B-fragment of diphtheria toxin.

Dim9, Dim25 and Dim27 are toxin neutralising in the order Dim9 = Dim25 > Dim27. The epitope for Dim27 is largely destroyed in diphtheria toxoid by the formaldehyde treatment [22]. Furthermore, Dim9 inhibits the binding of mouse antiserum to diphtheria toxin, indicating that the epitope is immunodominant [21].

Anti-DTd monoclonal antibody DiD1 was purchased from Abnova (Taiwan) and binds to the B-fragment of DTx and DTd.

Horse anti-DTd serum (PaD) and horse anti-DTd peroxidase conjugate (PaDPO) were obtained from BBio.

HRPO-conjugated goat-anti-mouse IgG was bought from Southern Biotech (Birmingham, USA).

2.3. Adsorption of DTd-X, DTd-Y and DTx to Adju-Phos

Experimental vaccines adDTd-X, adDTd-Y and adDTx were prepared by adsorbing 15 Lf/ml of DTd-X, DTd-Y or DTx, respectively, to 0.33 mg/ml Al^{3+} (Adju-Phos 2%, Brenntag, Denmark) (Table 1). The vaccines were incubated by stirring overnight (o/n) at 4 °C, and then stored at 4 °C prior to the analysis.

2.4. Desorption of DTd-X, DTd-Y and DTx

Desorption of DTd or DTx from aluminium phosphate was achieved by adding 5% (w/v) sodium citrate (Calbiochem, Merck Germany) and incubating the vaccines o/n at 37 °C.

2.5. Sandwich ELISA for pre-adsorbed and desorbed DTd and DTx

Polystyrene 96-well plates (Greiner Bio-one, Germany), were coated overnight at room temperature with PaD (0.6 AU/ml) in 0.4 M carbonate buffer, pH9.6. The plates were washed with tap-water containing 0.05% Tween 80. DT79/1 and test samples were titrated using twofold dilutions series in buffer A (phosphate-buffered saline (PBS, pH 7.4), containing 0.5% Protifar (Nutricia, the Netherlands) and 0.05% Tween 80 (Merck, Germany), starting at 1.2 Lf/ml. Following incubation for 2 h at 37 °C, the plates were washed. Epitope-specific mabs or polyclonal antibodies were added (Dim9 1:8000; DiD1 1:4000; Dim24 1:1000, Dim25 and Dim27 1:2000; and PaDPO 1:2000 in buffer A) and the plates were incubated 1.5 h at 37 °C. After washing the plates, HRPO-conjugated goat-anti-mouse IgG (1:2000 in buffer A; Southern Biotech) was added to each well, except for the wells containing PaDPO, followed by incubation at 37 °C for 2 h. Plates were washed and tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added to all wells. After 10 min the reaction was stopped by addition of 0.2 M H_2SO_4 and absorbance at 450 nm was measured. Antigen content was calculated relative to DT79/1 by parallel line analysis (log optical density vs. log dose), using a minimum of 3 sequential points from the linear section of the dose-response curve for the control and test sample.

2.6. Immunoassay for aluminium-adsorbed DTd and DTx

The immunoassay previously described by Zhu et al. [18] was used with some modifications. Test samples were added in