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Induction of *Bordetella pertussis*-specific immune memory by DTPa vaccines

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ABSTRACT

Several vaccines are available against pertussis, differing by the number of *Bordetella pertussis* antigens that they contain as well as their formulation. The GlaxoSmithKline Biologicals (GSK Bio) tricomponent DTPa vaccine (DTPa3, *Infanrix*™), and the Sanofi-Pasteur (SP) five-component formulation (DTPa5, *Pediacel*™) were shown to have comparable short-term efficacy in clinical trials. However, potential differences in long-term protection were recently suggested, which might reflect the elicitation of different specific immune memory by the two vaccines. Therefore, the purpose of the present study was to investigate in mice the immune responses against *B. pertussis*, and particularly the establishment of specific B cell memory after immunization with DTPa3 and DTPa5 vaccines. Whereas intranasal challenge experiments showed similar protection with both vaccines, DTPa3 induced higher antibody levels to FHA and PRN than DTPa5. Further, the frequency of memory B cells was investigated by B cell ELISPOT. Higher frequencies of PT- and PRN-specific memory B cells were evidenced after vaccination with DTPa3, compared with DTPa5. Although the origin of such difference is unclear, the use of two different adjuvants (aluminum phosphate versus hydroxide) is proposed as a possible explanation. In conclusion, this study proposes that the induction of higher levels of *B. pertussis* antigen-specific memory B cells with DTPa3 participate to the suggested longer persistence of protection observed with this vaccine, as compared with DTPa5.

1. Introduction

Bordetella pertussis is a gram-negative bacterium, known to be the responsible agent of whooping cough in humans. It attaches to and damages the respiratory epithelium, causing impairment of the normal respiratory activity, leading to serious complications and even death [1]. Vaccination has proven the best strategy to control the disease. As early as in the 1940s and the 1950s, whole-cell pertussis (Pw) vaccines were introduced in many countries, combined early on with tetanus toxoid (TT) and diphtheria toxoid (DT). In countries with high vaccine coverage, a striking decrease in pertussis-induced morbidity and mortality, up to very low levels, was observed [1,2]. This success was somewhat tarnished by safety concerns about the Pw vaccines, which motivated the development of acellular pertussis (Pa) vaccines, first in use in Japan, then in Europe and the USA since the 1990s.

Pa vaccines are composed of purified *B. pertussis* proteins, particularly pertussis toxoid (PT), filamentous hemagglutinin (FHA), pertactin (PRN), and the fimbrial agglutinogens FIM2 and FIM3. These antigens are often combined with TT and DT. Conversely to diphtheria and tetanus, for which a single vaccine antigen is needed, efficient protection against pertussis seems to necessitate the presence of at least three antigens in the formulation. Indeed, analysis of clinical studies of Pa vaccines showed that the efficacy of three- or more component vaccines is comparable to that of Pw vaccines, whereas one- and two-component vaccines were found less efficacious [3–5]. Similar observations were made in mouse intranasal challenge experiments [6,7]. Noteworthy, although the efficacy of Pa vaccines with more than three antigens was demonstrated, no correlate of protection could be clearly established. Nevertheless, several clinical studies found a link between protection against pertussis and the levels of IgG antibodies to PRN, FIM2, or PT [8–12], which suggested a critical role for the antibodies in protection. Follow-up studies of vaccinated cohorts, however, evidenced decline in these specific antibody levels, particularly for anti-PT, dramatic enough in some cases to become undetectable two years after the completion of Pa vaccination [13,14], while, in contrast, the protection afforded was reported to last 4–12 years [15,16]. A similar observation was made in a preclinical study, in which it was shown that vaccinated animals were still protected 44 weeks after immunization despite waning of *B. pertussis*-specific IgG down to undetectable levels [17].

When long-term protection was evaluated in follow-up studies in Sweden, a trend of more vaccine failures among DTPa5 (acellular pertussis vaccine containing five antigens) recipients versus

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DTPa3 (with three antigens) recipients was observed [18], and further confirmed [19]. This observation reveals potential differences in long-term protection between the two types of vaccines, namely the GlaxoSmithKline Biologicals (GSK Bio) tricomponent DTPa (*Infanrix*™/*Pediarix*™), containing PT, FHA, and PRN as *B. pertussis* antigens, and the Sanofi-Pasteur (SP) five-component formulation (*Pediacel*™/*Pentacel*™), with PT, FHA, PRN, FIM2 and FIM3. The two vaccines have a different composition but they also are formulated with a different adjuvant. In *Pediacel*™, the antigens are adsorbed on aluminum phosphate, whereas they are adsorbed on aluminum hydroxide in *Infanrix*™. Such difference in adjuvantation was held responsible for the lower humoral response and poorer protection against intranasal challenge in mice which was observed after immunization with aluminum phosphate-adsorbed, compared with aluminum hydroxide-adsorbed PT, FHA and PRN [20]. Nevertheless, these two vaccines were shown to have comparable efficacy in clinical trials [3,4,18,21] but this claim resulted from the evaluation of short-term immune responses only.

Long term protection against pertussis can be mediated by antibodies. This idea is supported by the fact that the level of antibody against some pertussis antigens seems to play a role to prevent the disease [8–11]. T cell immunity may also play a role [17,22]. In addition, long term protection against pertussis can also be associated with the presence of specific memory B cells. Besides the generation of long-lived plasma cells that play a key role in maintaining long term circulating antibody titers, memory B cells are the second component of the immunological memory generated by infection or vaccination. Memory B cells do not produce antibodies, but recirculate through blood and are endowed upon encounter of their cognate antigen with the capacity to differentiate quickly into plasma cells, secreting already class-switched high-affinity antibodies. The generation of memory B cells has been demonstrated for various vaccines [23–26]. Besides a clear role in the response to booster immunization, they could play diverse roles in protection, depending on the pathogen considered. Memory B cells may be directly involved in the protection against pathogen invasion in the absence of high levels of circulating antibodies, as demonstrated for hepatitis B [27]. Alternatively, memory B cells may help to replenish the pool of long-lived plasma cells, hence ensuring the continuous production of antibodies, as was recently demonstrated [28]. Of note, another recent study [29] demonstrated that this differentiation of memory B cells into plasma cells is only achieved upon stimulation with cognate antigen rather than by bystander inflammatory signals, conversely to what was previously suggested [30].

Therefore, the observations in the Swedish follow-up studies might reflect the elicitation of different specific immune memory by the two vaccines. In order to link the field observations with immune parameters, the purpose of the present study in mice was to investigate the B cell responses against *B. pertussis*, and particularly the establishment of the specific B cell memory, after immunization with DTPa3 and DTPa5 vaccines.

2. Materials and methods

2.1. Mice and study schedules

All experiments and assays were performed at GlaxoSmithKline Biologicals (GSK, Rixensart, Belgium) in accordance with the Belgian national guidelines for animal experimentation. Groups of 20 BALB/c mice (females, 5-week old) were used. The mice received injections of DTPa5-IPV-Hib (*Pediacel*™), DTPa3-IPV-Hib (*Infanrix*™-IPV-Hib), DTPa3 (*Infanrix*™) or DTPa3-IPV-HBV-Hib (*Infanrix hexa*™), depending on the group. *Infanrix*™, *Infanrix*™-IPV-Hib and *Infanrix hexa*™ can be used indifferently in the intranasal challenge model since they have been shown to afford the same protection [31]. Animals in control group were not vaccinated. At day 0, the animals were injected 125 μl of vaccine (¼ of a human dose), followed by a booster two weeks later (day 14). All vaccines were administered subcutaneously in the upper back without further dilution. Intranasal challenge was carried out at day 28. For B cell memory investigations, groups of 20 mice were each divided into 10 pools of 2 animals. Spleens were analyzed for memory B cells at day 41 in animals that have not been challenged. For the global statistical analysis, two additional experiments were taken into account, where groups of 12 mice were used, divided into 6 pools of 2 mice.

2.2. ELISA to B. pertussis antigens

One day before intranasal challenge (day 28), serum samples were collected from all mice. ELISA microplates were coated overnight at 4 °C with PT, FHA, or PRN (2, 8, and 6 μg/ml, respectively, in bicarbonate buffer 50 mM, pH 9.6). The antigens PT, FHA, and PRN were those included in the acellular vaccine in their native state, i.e. not chemically inactivated. Then, wells were saturated in PBS-BSA 1% before serum samples (diluted 1/100 in PBS-BSA 1%, Tween-20 0.05%) were added. Bound antibodies were detected by incubation for 30 min at room temperature in peroxidase-conjugated anti-mouse IgG (GE Healthcare), diluted 1/2000 in PBS-BSA 1%; Tween-20 0.05%, followed by the peroxidase substrate o-phenylenediamine dihydrochloride in citrate buffer 0.1 M, pH 4.5 in the presence of hydrogen peroxide. The reaction was stopped by the addition of HCl 1 N, and the plates were read at 490 and 620 nm. Extensive washings with NaCl 150 mM-Tween 20 0.05% were carried out between each step. Normal mouse serum pool and hyperimmune DTPa-sensitized serum were used as lower and upper reference, respectively. The protocol was identical for the detection of the isotypes IgG1 and IgG2a, except that the coating for FHA was made at 2 μg/ml. Bound IgG1 and IgG2a were detected by peroxidase-conjugated goat anti-mouse IgG1 (Accurate Chemical & Scientific) and peroxidase-conjugated goat anti-mouse IgG2a (Accurate Chemical & Scientific), respectively.

2.3. Intranasal challenge

The *in vivo* mouse *B. pertussis* lung clearance assay is based on the analysis of the lung invasion by *Bordetella* strains following standard sub-lethal intranasal challenge of vaccinated mice [32].

Two weeks after the second vaccine injection, mice were challenged by instillation of 50 μl of bacterial suspension (strain 18323) (equivalent to a total of approximately 5×10^6 colony forming units (cfu)) into the left nostril under light isofurane anesthesia. For the intranasal challenge models, bacterial suspensions of *B. pertussis* grown on Bordet–Gengou agar and in Stainer–Scholte liquid medium were diluted in the latter to provide a challenge inoculum of approximately $1 \times 10e^8$ cfu/ml for a sub-lethal challenge. A 50 μl aliquot of bacterial suspension was slowly administered into the nostril using a micropipette so as to be immediately aspirated by the animal respiration. After the challenge, 5 infected mice per time point were sacrificed by anesthesia 2 h after exposure (designated day 0) and on the days 5 and 8. The lungs were removed, homogenized in 2 ml of casaminoacid (1%)-buffered saline with tissue grinders, and 10-fold serial dilutions of the homogenates plated on BGA and incubated at 37 °C for 4–6 days before cfu counting. The $\log_{10}$ weighted mean number of cfu per lung (cfu_w/lung) and the standard deviation were calculated for each time point.

2.4. B cell ELISPOT

At day 41, spleens were removed and placed in RPMI containing additives (L-glutamine (2 mM), non-essential amino acids