

Original article

Alternative diphtheria, tetanus and whooping cough immunization schedule to evoke a Th2 tetanus and a Th1 pertussis immune response

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Abstract

In a previous study, using BALB/c mice, we found that while diphtheria (D), tetanus (T) and whooping cough (Pw, whole-cell *Bordetella pertussis*) immunization induces a Th1/Th2 tetanus response and memory T cells able to proliferate in response to in vitro stimulation with *B. pertussis*, DTPa immunization induces a Th2 tetanus immune response and no memory T cells that recognize *B. pertussis* as stimulus. Considering that a pro-inflammatory cytokine production is not necessary for protection against tetanus and therefore should be avoided, an alternative DTP immunization schedule with minimal Pw exposure was assessed in order to obtain a Th2 tetanus response and a Th1 pertussis response. BALB/c mice were primed with DT vaccine at day 0, with Pw vaccine at day 14 and boosted with DTPa vaccine at days 21 and 28. A control group was inoculated with saline. Antibodies against *B. pertussis* surface antigens, tetanus and diphtheria toxoids were produced by mice. Spleen cells stimulated in vitro with *B. pertussis* produced IL-6 and IFN γ . Only IL-5 was produced by cells in response to tetanus toxoid stimulation. These results are in line with the low IgG1/IgG2a ratio for pertussis antibodies compared with those corresponding to tetanus and diphtheria. The immunization protocol presented herein succeeded in producing tetanus and pertussis immune responses of Th2 and Th1 type, respectively. In contrast to previous results obtained with DTPw immunization, no IL-12 production was observed. Our findings provide direct evidence that an immunization protocol with an interval of 14 days between DT and Pw primings, followed by DTPa boosters, can induce appropriate immune responses against DTP vaccine antigens.

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Keywords: DTP vaccination; Tetanus immune response; Alternative immunization schedule

1. Introduction

Because of the increasing number of vaccines now routinely recommended for infants, the development of combined vaccines that enable simultaneous administration, with fewer separate injections, is a high priority.

The Argentine population is immunized against tetanus, diphtheria and whooping cough by DTPw vaccination (diphtheria (D) and tetanus (T) toxoids and whole-cell *Bordetella pertussis* (Pw)) at 2, 4, 6 and 18 months of age. Booster doses of tetanus and diphtheria toxoids are recommended at the age of 16 and every 10 years. A person can receive additional doses in certain situations, e.g. accident, surgery, etc. Although the DTPa vaccine (D and T toxoids and soluble *B. pertussis* antigens (Pa)) is less reactogenic than the DTPw vaccine, it is not widely used in Argentina because of its high price.

Using a murine model (BALB/c mice), we analyzed the tetanus immune response elicited by DTPw, DTPa and DT vaccination and found that while vaccines composed of soluble antigens induce an immune response of a Th2 type, DTPw vaccine induces a mixed (Th1/Th2) immune response with the secretion of pro-inflammatory cytokines that are not involved in protection against tetanus. We also showed that in contrast with DTPw immunization, DTPa immunization does not induce memory T cells able to proliferate to in vitro stimulation with *B. pertussis* [1].

To design an alternative immunization protocol to induce a Th2 type immune response to tetanus and diphtheria toxoids and a Th1 immune response to *B. pertussis*, we considered the following:

- (a) unnecessary pro-inflammatory cytokine production should be avoided because it could be harmful to susceptible individuals [2–8];
- (b) humoral immune responses are necessary for protection against tetanus as well as against diphtheria;

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- (c) cellular immune responses, in particular, effector mechanisms mediated by inflammatory cytokines, are required to eliminate an intracellular pathogen such as *B. pertussis*;
- (d) priming determines the type of immune response that will be evoked with booster doses.

2. Materials and methods

2.1. Mice

Female BALB/c mice (6–8 weeks old) were provided by the INTA (Instituto Nacional de Tecnología Agropecuaria, Argentina).

2.2. Vaccines

The vaccines used were as follows. Pw vaccine: adsorbed *B. pertussis* (2.96×10^{10} cells/ml) (The Human Serum Production and Medicine Manufacturing Company Limited). DT vaccine: adsorbed tetanus (10 Lf/ml) and diphtheria (30 Lf/ml) toxoids (Connaught Lab. Ltd., Toronto, Ontario). DTPa vaccine: adsorbed tetanus (10 Lf/ml) and diphtheria (30 Lf/ml) toxoids combined with a mixture of pertussis toxoid (20 µg/ml), filamentous hemmagglutinin (10 µg/ml), fimbriae (AGG 2 + 3, 10 µg/ml) and pertactin (6 µg/ml) (Connaught Lab. Ltd., Toronto, Ontario). Vaccines were diluted 1:2.5 in sterile saline.

2.3. Immunizations

The immunization schedule consisted of DT priming at day 0, Pw priming at day 14 and DTPa boosters at days 21 and 28. Groups of five mice each were injected with 0.5 ml of saline as control or immunized with 0.5 ml of the vaccine dilution given in two subcutaneous injections.

2.4. T cell responses

Ten days after the last immunization, individual spleen cell suspensions of each immunized mouse or control were prepared in culture medium (complete RPMI-1640 containing 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 300 µg/ml L-glutamine and 220 µg/ml pyruvate). One hundred microlitres of spleen cell suspension (4×10^6 cells/ml) was cultured with 100 µl of culture medium or with 100 µl of antigen in quadruplicate at 37 °C in the presence of 5% CO₂. Antigens tested were tetanus toxoid (10 µg/ml; Statens Serum Institut, Copenhagen) and heat-killed *B. pertussis* (4×10^6 cells/ml; Statens Serum Institut). Concanavalin A (ConA, 5 µg/ml; Vector Labs., Burlingame, CA) was used as mitogen. After 72 h, supernatants were removed and stored at –70 °C until assayed for cytokine levels. Proliferative responses were assessed after 96 h of culture by [³H] thymidine

uptake and measured by liquid scintillation counting, and the results were expressed as mean count per minute for quadruplicate cultures.

2.5. Cytokine assays

Commercial kits were used to determine levels of IL-5, IFNγ, IL-6 and IL-12 (PharMingen, San Diego, CA).

2.6. Levels of serum antibodies

Before removal the spleen, each animal was bled by retro-orbital puncture. Individual serum samples were separated and stored at –20 °C until assay for antibody levels. Tetanus, diphtheria and pertussis antibody levels were measured by ELISA.

To determine the isotype of specific antibodies, HRP-conjugated anti-mouse IgG1 and IgG2a sera (Bethyl Laboratories Inc., Montgomery, TX) were used. Before obtaining the IgG1/IgG2a ratio, all sera were diluted to obtain the same total specific IgG levels.

2.7. Statistical analysis

To compare antibody levels, IgG1/IgG2a ratios, proliferation and cytokine data, one-way ANOVA was used.

3. Results

Stimulation with ConA invariably induced proliferation in all groups of mice. All immunized animals responded to in vitro stimulation with tetanus toxoid and with *B. pertussis* ($P < 0.001$) (Fig. 1).

IFNγ and IL-5 were the cytokines chosen as indicators of Th1 and Th2 type immune responses, respectively. Cells from all immunized animals produced IL-5 in response to tetanus toxoid stimulation ($P < 0.001$) (Fig. 2). The immunization protocol induced the production of IFNγ by cells stimulated with *B. pertussis* ($P < 0.001$) (Fig. 3). IL-6, a cytokine associated with inflammatory processes, was produced only by primed cells stimulated with *B. pertussis*

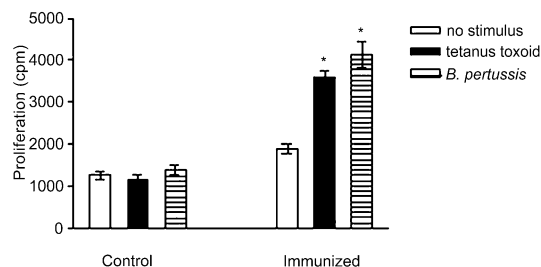


Fig. 1. Immunized mouse spleen cell proliferation. Ten days after the last immunization, spleen cells were isolated and incubated with culture medium, tetanus toxoid or *B. pertussis* for 96 h. Five mice from each group were analyzed in each assay, and results are expressed as mean counts per minute (cpm). Bars represent results from two separate assays. * $P < 0.001$ by one-way ANOVA.

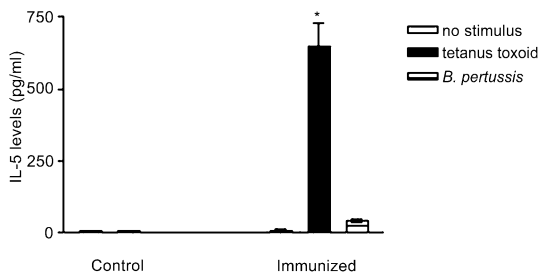


Fig. 2. IL-5 production. IL-5 levels measured in supernatants of spleen cells incubated with culture medium, tetanus toxoid or *B. pertussis* for 72 h. Five mice from each group were analyzed in each assay, and results are expressed in pg/ml. Bars represent results from two separate assays. * $P < 0.001$ by one-way ANOVA.

($P < 0.01$) (Fig. 4). IL-12 was chosen for its involvement in the activation of Th1 cells. No difference was observed between IL-12 levels when measuring culture supernatants of unstimulated primed and unprimed cells (Fig. 5).

Diphtheria and tetanus antibody titers observed were 5.6 ± 2.4 IU/ml and 44.8 ± 17.4 IU/ml, respectively. *B. pertussis* IgG antibody titers were 0.3 ± 0.1 EU/ml.

The *B. pertussis* IgG1/IgG2a antibody ratio (1.8 ± 1.3) was lower than the corresponding ratios for tetanus and diphtheria antibodies (3.2 ± 1 and 3.9 ± 1.2 , respectively, $P < 0.05$) (Fig. 6).

4. Discussion

A great deal of knowledge about host–pathogen interactions has been gathered over the last years. This has been

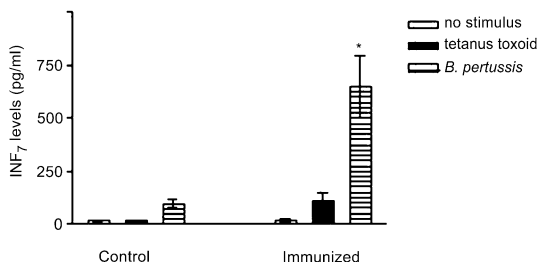


Fig. 3. IFN γ production. IFN γ levels were measured in supernatants of spleen cells incubated with culture medium, tetanus toxoid or *B. pertussis* for 72 h. Five mice from each group were analyzed in each assay, and results are expressed in pg/ml. Bars represent results from two separate assays. * $P < 0.001$ by one-way ANOVA.

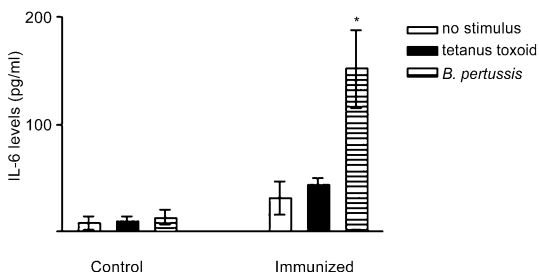


Fig. 4. IL-6 production. IL-6 levels were measured in supernatants of spleen cells incubated with culture medium, tetanus toxoid or *B. pertussis* for 72 h. Five mice from each group were analyzed in each assay, and results are expressed in pg/ml. Bars represent results from two separate assays. * $P < 0.01$ by ANOVA.

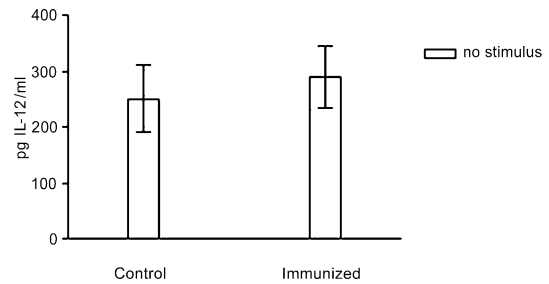


Fig. 5. IL-12 production. IL-12 levels were measured in supernatants of spleen cells incubated with culture medium for 72 h. Five mice from each group were analyzed in each assay, and results are expressed in pg/ml. Bars represent results from two separate assays.

useful for the development of new vaccines and has encouraged an insightful study of the modulatory effects of combined vaccines on the immune response they elicit. We studied the immune response of BALB/c mice immunized with DT, DTPw or DTPa vaccine and found that the association of vaccine antigens can modify the original immune response to each antigen administered alone, for we observed that the tetanus immune response is not of a Th2 type if whole-cell *B. pertussis* is present in the vaccine formulation [1]. Taking into account that the tetanus immune response is evoked several times during the lifetime, avoiding Th1 tetanus immune responses should be of interest, because this inflammatory response is not necessary for protection against tetanus and could be harmful in autoimmune-prone individuals [2–8]. In order to overcome this drawback, we designed a different DTP immunization schedule, in which a Th2 tetanus immune response and a Th1 pertussis immune response could be obtained.

The administration of only one Pw inoculum (the one mainly responsible for DTPw vaccination reactogenicity), together with DTPa boosters, enabled the induction of memory T cells able to proliferate in response to in vitro stimulation with *B. pertussis*. These memory T cells synthesized IFN γ in response to in vitro *B. pertussis* stimulation, which defines a Th1 profile, the type of immune response necessary to induce the clearance of an intracellular pathogen such as *B. pertussis* [9–12]. Immune responses of an inflammatory profile are also observed after DTPw priming and after *B. pertussis* infection [13,14]. Nevertheless, DTPa immunization does not induce memory T cells that can proliferate in response to in vitro stimulation with *B. pertussis* [1]. The immunization schedule presented herein succeeded in

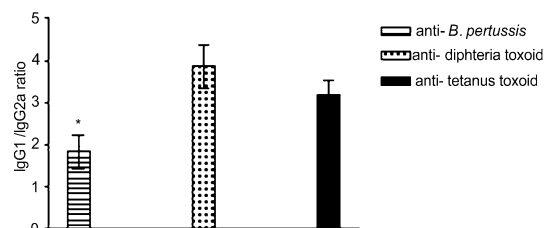


Fig. 6. IgG1/IgG2a antibody ratios. Pertussis, diphtheria and tetanus IgG1/IgG2a antibody ratios were determined in individual serum samples by ELISA. * $P < 0.05$ by one-way ANOVA.

producing Th1 pertussis and Th2 tetanus immune responses, while DTPw immunization elicited pertussis and tetanus immune responses of a mixed type (Th1/Th2). Considering this, an interval of 14 days between Pw and DT primings likely enabled the evading of the modulatory effect of DT on the *B. pertussis* immune response as well as the modulatory effect of Pw on the tetanus immune response. It is worth mentioning that an interval of 7 days between Pw and DT priming was not sufficient to obtain the same results (data not shown).

In contrast with previous results obtained with DTPw immunization, IL-12 was produced at basal levels (unstimulated cells). McGuirk and Mills [15] have recently shown that filamentous hemmagglutinin (FHA), a virulence factor of *B. pertussis*, has direct anti-inflammatory activity. Considering that FHA is one of the components of the DTPa vaccine used in this work, we think that this antigen may inhibit IL-12 production by macrophages.

With regard to the humoral immune response, antibodies against a variety of *B. pertussis* surface antigens were detected. The well-known immunogenicity of tetanus toxoid is in agreement with the high serum tetanus antibody titers obtained. Diphtheria antibody levels were similar to those obtained with DTPw and DTPa vaccines (data not shown). When comparing antibody isotypes, the low IgG1/IgG2a ratio for pertussis antibodies is in line with the IFN γ production by *B. pertussis*-stimulated cultured cells, since IFN γ induces the IgG2a switch [16,17].

Our findings using this immunization protocol provide direct evidence that priming with DT and Pw with an interval of 14 days, followed by DTPa boosters, can generate appropriate immune responses against DTP vaccine antigens. Future studies will be necessary to evaluate the protection status obtained after application of the immunization protocol presented herein.

Acknowledgements

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