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Microbes and Infection xx (2007) 1–5

www.elsevier.com/locate/micinf

Short communication

Neonatal immunization with a single dose of recombinant BCG expressing subunit S1 from pertussis toxin induces complete protection against *Bordetella pertussis* intracerebral challenge

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Received 25 April 2007; accepted 17 October 2007

Abstract

The currently used pertussis vaccines are highly efficacious; however, neonates are susceptible to whooping cough up to the sixth month. In agreement, DTP-immunized neonate mice were not protected against intracerebral challenge with *Bordetella pertussis*. Neonate mice immunized with either DTP or a recombinant-BCG strain expressing the genetically detoxified S1 subunit of pertussis toxin do not show a humoral immune response against PT. On the other hand, rBCG-Pertussis induces higher PT-specific IFN-γ production and an increase in both IFN-γ^+ and TNF-α^+-CD4$^+$-T cells than the whole cell pertussis vaccine and confers protection against a lethal intracerebral challenge with *B. pertussis*.

Keywords: Recombinant BCG vaccine; Pertussis; Neonates

1. Introduction

Pertussis, also known as whooping cough, is caused by *Bordetella pertussis*; the disease may strike all ages, but the early infants are the most vulnerable group. Of the 20–40 million people affected worldwide, approximately 1% dies each year, mostly those below 6 months of age [1]. Although the immunization schedules used world wide, in which infants receive 3 doses of DTP (diphtheria, tetanus and pertussis vaccine) before 6 months of age have been efficient in preventing or attenuating pertussis manifestations in children, young infants are still unprotected from birth up to the sixth month [1]. Furthermore, despite high vaccination coverage, a worldwide resurgence of *B. pertussis* infection has been observed, mostly in adolescents and adults, the age groups that play a significant role in transmission of pertussis to neonates and infants [2]. As pertussis continues to pose a significant disease burden, there remains a need to implement new vaccine strategies to enhance its control.

The pertussis component can be either the whole inactivated *B. pertussis* cells (as in DTP) or the acellular pertussis components (as in DTaP), containing up to five purified *B. pertussis* antigens; pertussis toxin is the main antigen in all formulations [3]. Both DTP versions, whole-cell and acellular, present high efficacy, but figures can vary depending on the source of the vaccine [3]. All in all, there has been a general agreement that DTaP vaccines are efficacious and less reactogenic [3]; however, their high financial cost limits their

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doi:10.1016/j.micinf.2007.10.010

implementation in the developing world [4]. Furthermore, DTP (or DTaP) vaccination in neonates has not been implemented, allegedly, due to immaturity of the immune system, which limits protection induced by the vaccine in early infants [4–6].

The live attenuated *Mycobacterium bovis*-Bacille Calmete Guérin (BCG) vaccine, in turn, has been given to newborns as a safe vaccine to prevent severe and fatal tuberculosis, following WHO recommendations [7]. Moreover, BCG is able to elicit T helper-type 1 (Th1) immune responses to its own antigens and to other vaccines given in early life, also acting as a potent immune adjuvant [8]; Th1 immune responses are important protective mechanisms against pathogens such as *B. pertussis* [9]. Therefore, we have chosen BCG as a system for presenting pertussis antigens, in an attempt to achieve immunization against pertussis in newborns. We have previously constructed a recombinant BCG strain expressing the S1 subunit of the genetically detoxified *B. pertussis* toxin, PT-9K/129G [10]. Adult mice immunized with the so called rBCG-S1PT strain displayed high levels of protection upon intracerebral challenge with live *B. pertussis* [11].

In the present work, we took advantage of the recently established mouse model for investigating immunization in neonates, in which an immunological correlation is proposed between human and mouse; that is, the neonatal period of humans – defined as 28 days – may correspond to that of a one-week-old mouse [12]. Therefore, we have immunized neonate mice with the rBCG-S1PT strain to evaluate the induced humoral and cellular-type immune responses and survival frequency following intracerebral *B. pertussis* challenge.

2. Materials and methods

2.1. Bacterial strains

Mycobacterium bovis BCG Moreau strain (Instituto Butantan) and rBCG-S1PT – a recombinant BCG strain expressing the S1 subunit of the detoxified Pertussis toxin (S1PT) fused to the signal sequence of the β-lactamase gene were prepared as previously described [11].

2.2. Neonate mice immunization

Groups comprising litters of 8–15 newborn outbred Swiss mice (Instituto Butantan, SP, Brasil) were immunized with 50 μl of one-tenth dose of: (i) an adult mouse dose (which is 1/8th of a human dose) of DTP (whole-cell), given at day 5 (and / or day 12 – as specified); or $\sim 1 \times 10^5$ colony forming units (CFU) (1/10th adult mouse dose) of (ii) BCG; or (iii) rBCG-S1PT, on day 5 according to the different regimens.

2.3. ELISA and Lymphokyne assays

Sera from immunized mice were obtained before challenge and analyzed for total IgG induced against detoxified pertussis toxin (dPT), supernatant-derived proteins of BCG (SDP) or tetanus toxoid (TT) as determined by enzyme-linked immunosorbent assay (ELISA) [12]. Splenocytes (2×10^6/ml) obtained from three mice, 16 days after immunization were cultured in the presence or absence of dPT (1 μg/ml) as the antigen-specific stimulation. The culture supernatants were harvested at 48 h for quantification of gamma interferon (IFN-γ) by ELISA [12].

2.4. FACS analysis of T cell phenotypes and intracellular staining of TNF-α and IFN-γ-secreting T cells

Immunostaining and FACS analysis were carried out by using standard procedures. Splenocytes obtained from three mice, 16 days after immunization were subjected to flow cytometric analysis. The phenotypes of T cells were examined using mAb's against murine CD8 (rat; FITC label), and CD4 (rat; CyChrome label). Data were collected by FACScan (Becton Dickinson Immunocytometry Systems, Sunnyvale, California, USA) with gating on the lymphocyte region, and analyzed using FlowJo software (Eva Haberfeld, USA). For intracellular staining of TNF-α and IFN-γ, isolated total splenocytes were cultured for 6 h in the presence of brefeldin (GolgiPlug) with or without anti-CD3 mAb (5 μg/mL). Cells were then processed for staining with mAbs for CD4 (Rat; CyChrome label), CD8 (rat; FITC) and a phycoerythrin-conjugated anti-murine TNF-α or IFN-γ antibody (rat; phycoerythrin). All of the reagents used for FACS and intracellular staining were purchased from BD Pharmingen (San Diego, California, USA).

2.5. B. pertussis challenge

Immunized mice were subjected to intracerebral (i.c.) inoculation with a lethal dose of *B. pertussis* 18323 suspension containing approximately 3×10^4, 3×10^5 or 9×10^5 CFU (in 30 μl), on day 21st after birth (when mothers were withdrawn). The number of survivors was monitored up to 12 days after challenge and the level of protection was expressed as the percentage of live animals.

2.6. Statistical analysis

Student's *t* or Fischer tests were employed to determine the significance of differences between the studied groups. *p*-values of <0.05 (*) were considered significant.

3. Results

3.1. Immunization of neonate mice with DTP does not protect against intracerebral challenge with B. pertussis

Mice immunized with one dose of DTP, either at day 5 or 12 after birth, showed low protection levels: 30 and 43%, respectively (Table 1). It was only with a double-dose vaccine scheme (days 5 and 12) that a significant protection level (75%) was observed ($p = 0.016$) (Table 1). It is interesting to note that we had to increase at least 10-fold the adult-mouse

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Table 1
Survival of DTP-immunized neonate mice after *B. pertussis* intracerebral challenge

Groups[a] (day of dose after birth)	No of survivors/ total mice	Survival (%)	*p*
Non-immunized	0/5	0	–
DTP (day 5)	3/10	30	0.26
DTP (day 12)	3/7	43	0.16
DTP (days 5 and 12)	6/8	75	0.016

[a] Neonate mice were immunized with DTP (1/10 the dose for adult mice) at the specified days of age (5 and / or 12); intracerebral challenge with a lethal dose, 3×10^5 CFU of *B. pertussis*, was carried out on the 21st day after birth and survival was monitored throughout the following 12 days. *p*, differences between the non-immunized and DTP groups were evaluated by the Fischer test.

dose of bacterial challenge to obtain a significant lethal effect in the early infant mice.

3.2. Immune response induced in neonate-immunized mice

Sera obtained from the DTP-immunized group on day 21st showed high titers against the positive control, TT, but no anti-dPT antibodies were detected (data not shown). For the rBCG-S1PT-immunized group, no anti-dPT antibody was detected, as expected in view of our previous results in adult mice [11]. However, sera from both BCG- and rBCG-S1PT-immunized animals showed no antibodies against mycobacterial antigens, SDP (data not shown), in contrast to what is observed in adult mice.

Splenocytes from mice immunized with rBCG-S1PT showed a statistically significant increase in dPT-stimulated IFN-γ production as compared to the non-immunized control ($p = 0.003$), which was also significantly higher than that induced by DTP ($p = 0.048$) (Fig. 1A). The BCG vaccine also elicited a considerable response, which can be attributed to a non-specific adjuvant effect of the mycobacterial components, as we have previously observed in adult mice [11].

We further investigated the expression of IFN-γ and TNF-α (intracytoplasmic) in splenocyte $CD4^+$ and $CD8^+$-T cells by flow cytometric analysis. Immunization with rBCG-S1PT induced a 3.2-fold higher percentage of $CD4^+$-T cells primed to produce IFN-γ as compared to immunization with DTP (not shown). The number of $CD4^+$-T cells capable of producing IFN-γ in animals immunized with rBCG-S1PT or BCG was 3.2–5-fold that induced by DTP (Fig. 1B). The number of $CD8^+$-T cells primed to produce IFN-γ was very low and the rBCG-S1PT group was comparable to DTP. On the other hand, $CD4^+$-T cells from rBCG-S1PT-immunized animals showed a 2.2-fold increase in the number of cells capable of producing TNF-α, which is higher than that induced by DTP or BCG immunization (1.4 and 1.5, respectively).

3.3. Protection upon intracerebral challenge

A single-dose immunization with rBCG-S1PT at day 5 led to 100% survival of mice, while DTP showed no protection (Fig. 2A). Nonetheless, a very high non-specific effect of BCG was also observed (80%). We have previously reported a protective effect of BCG alone in adult mice [11], although at lower levels (40%). At a higher challenge dose, even with a dose of DTP administered on day 12, this effect of BCG was reduced (50%) (Fig. 2B), also indicating that DTP does not boost the BCG non-specific effect. The challenge dose was further increased and the non-specific protection induced by BCG alone dropped to 11%. Even at this extremely high challenge dose (300-fold the lethal adult dose), immunization with rBCG-S1PT still induced 50% survival of neonate-immunized mice (Fig. 2C).

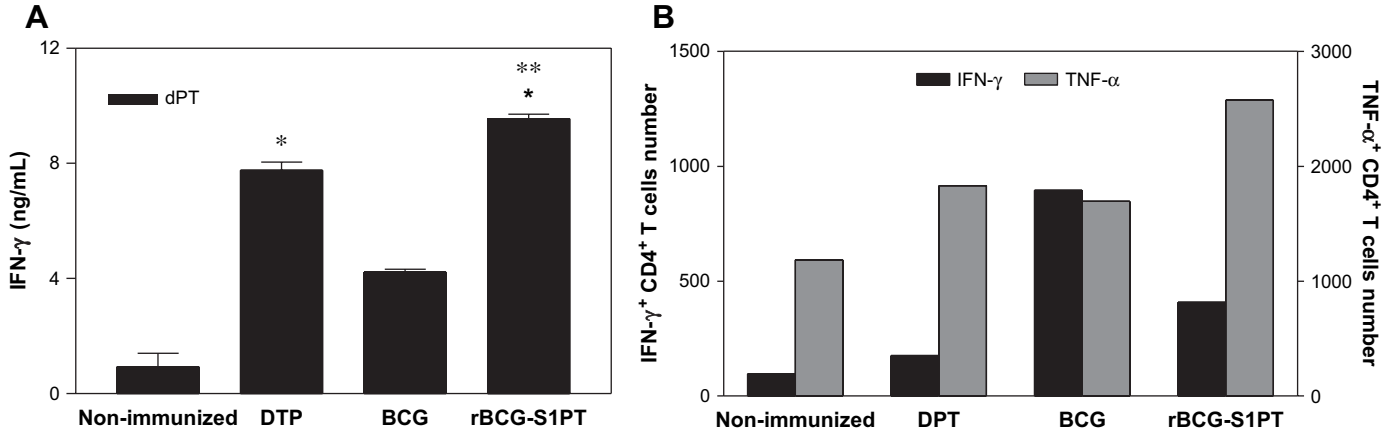

Fig. 1. Cytokine secretion of IFN-γ and analysis of $CD4^+$-T cells expressing IFN-γ and TNF-α by splenocytes from mice immunized with DTP, BCG or rBCG-S1PT. Spleen cells were isolated 16 days post-immunization, pooled and **A**) Stimulated with dPT antigen. $*p = 0.022$, for IFN-γ production from splenocytes of DTP-immunized animals as compared to non-immunized controls. $*p = 0.003$ and $**p = 0.048$, for rBCG-S1PT as compared with non-immunized and DTP-immunized animals, respectively. IFN-γ values are given as the means + the standard deviation. Statistical analysis was performed by the Student's *t* test. **B**) Cultured for 6 h in the presence of brefeldin with or without anti-CD3 mAb, and the cells subjected to intracellular TNF-α and IFN-γ staining and FACS analysis in conjunction with the use of anti-CD4 antibodies. The cell numbers represents the cell population that coexpresses both TNF-α and CD4 or IFN-γ and CD4.

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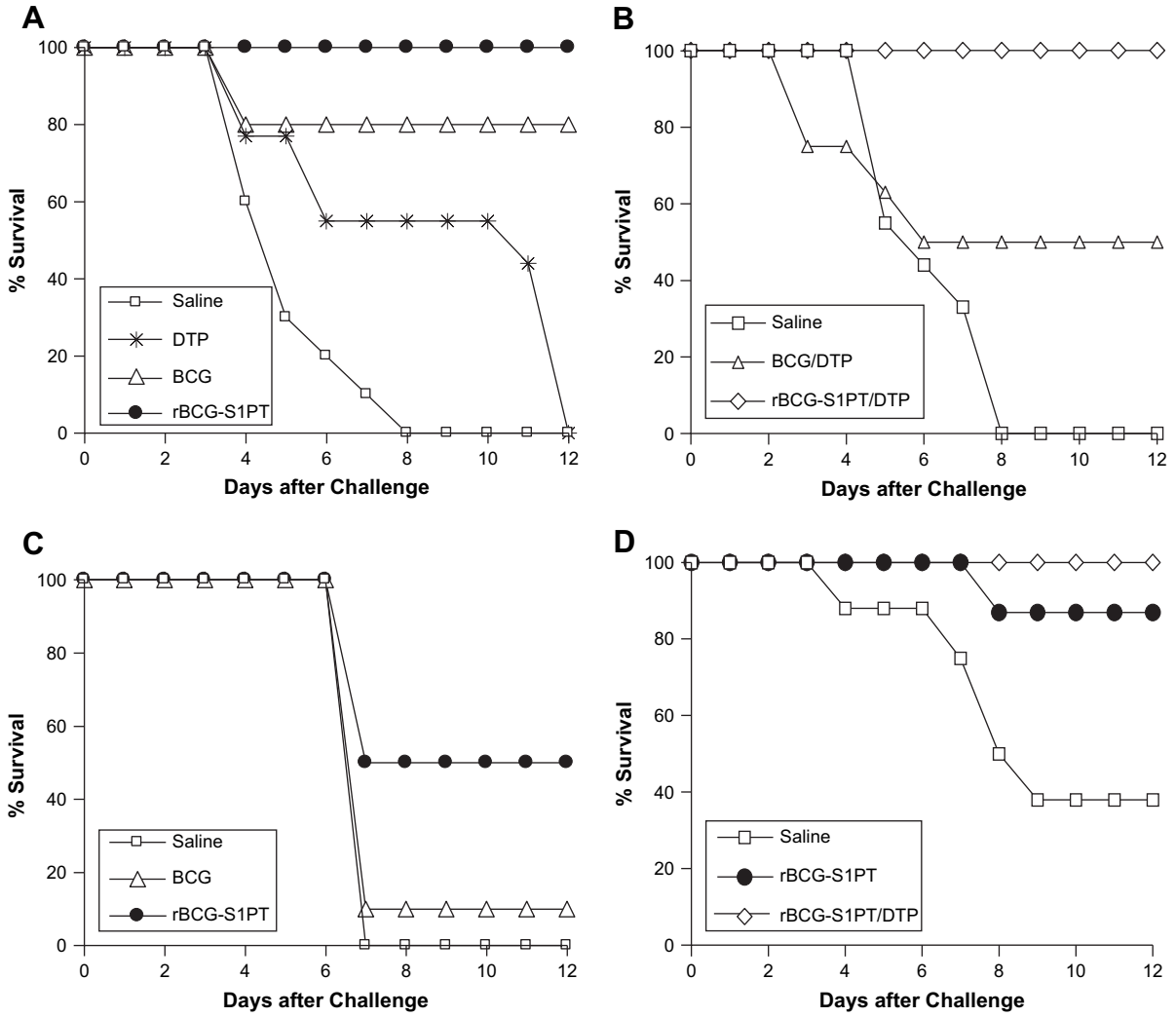

Fig. 2. Protection of neonate mice immunized with rBCG-S1PT against intracerebral challenge with *B. pertussis*. Groups of 5-days old neonate mice were immunized with DTP, BCG or rBCG-S1PT. Some groups (B and D) received a dose of DTP at day 12, as specified. On the 21st day, *B. pertussis* challenge was administered as a bacterial suspension containing: (**A**) 3×10^4, (**B**) 3×10^5, (**C**) 9×10^5 or (**D**) 3×10^4 CFU. Survival was monitored throughout 12 days after challenge.

On one occasion, rBCG-S1PT immunization showed a slightly lower protection level of 87% (Fig. 2D). Interestingly, in this case, a DTP dose given on day 12 complemented protection up to 100% (Fig. 2D). This complementation should not be due to the sum of the non-specific protection observed with BCG alone (Fig. 2A) and the low protection observed with DTP administered at day 12 (Table 1), since in the case group immunized with BCG/DTP, survival was no more than 50% (Fig. 2B).

4. Discussion

Immunization of early infant mice (21-day old) with DTP or DTaP has been shown to induce a reduction in bacterial load in the pertussis intranasal challenge model [12]; however, this does not correlate with what is observed in humans [1]. On the other hand, although the intracerebral challenge is the currently used method for certification of whole cell pertussis vaccines [13], it has not been used to evaluate neonate or early infant mice immunization with DTP. Therefore, we initially validated our experimental model showing that neonate mouse immunization with DTP did not protect them against an intracerebral challenge with *B. pertussis*. These results are in agreement with what is observed in human neonates [1], sustaining the predictive potential of using neonate mice for the prospective evaluation of pertussis vaccine efficacy in early infants.

Neonate mice immunized with DTP showed a reduced capacity to induce a humoral immune response against DTP antigens, which is in accord with previous reports of low antibody responses to PT in neonate and early infant mice immunized with whole-cell DTP, contrary to that observed for DTaP [12]. Furthermore, they observed that mice immunized with DTP or DTaP on day seven after birth and boosted three weeks later presented a low T cell response against PT. In the case of DTaP, it is not surprising, since the vaccine is known to induce mainly a humoral immune response in mice or in humans. However, the induction of a low cellular immune response

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by DTP contrasts with our results. It is important to note that there are several differences in the experiments; besides using different mouse strains, we are actually working with younger-aged mice and a much lower DTP dose. On the other hand, T-cell immune responses were seen in two month-old children, following *B. pertussis* acute infection, which would be in agreement with our results [14]. In our system, neither DTP nor rBCG-S1PT induced a humoral immune response against the pertussis component in the early infant mice, but splenocytes from mice immunized with rBCG-S1PT induced a significantly higher PT-specific IFN-γ production than that induced by DTP.

Here we show that immunization of neonate mice with rBCG-S1PT vaccine, contrary to DTP, is able to fully protect early infant mice against a lethal *B. pertussis* challenge. However, the difference in dPT-stimulated IFN-γ production between DTP and rBCG-S1PT should not account for the striking difference observed in protection. To evaluate the importance of T helper immune responses in the control of *B. pertussis* infection, we investigated the intracytoplasmic IFN-γ production by $CD4^+$ and $CD8^+$-T cells. We also measured TNF-α production, an inflammatory cytokine that appears to be important in the control of *B. pertussis* infection by limiting the neutrophil recruitment induced by PT [7]. Interestingly, DTP immunization induced very low IFN-γ^+-$CD4^+$-T cells, indicating that the IFN-γ observed in dPT-stimulated splenocytes should be produced by other cell types. These results would indicate that the production of IFN-γ by $CD4^+$-T cells is important for protection. TNF-α^+-$CD4^+$-T cells induced by DTP were comparable to naïve or BCG-immunized animals. Although BCG induced high levels of IFN-γ^+-$CD4^+$-T cells, a high proportion of these should not be PT-specific. Immunization with rBCG-S1PT was the only group to induce an increase in both IFN-γ^+ and TNF-α^+-$CD4^+$-T cells. On a whole, our results indicate that increased production of both cytokines by $CD4^+$-T cells is important for protection against *B. pertussis* infection.

Although it has long been proposed that recombinant BCG vaccines would have the potential of inducing protective immune responses in early life [15], to our knowledge, this is the first report in which protection induced by neonate immunization of mice with a recombinant BCG vaccine is actually demonstrated. Furthermore, our results indicate that after priming the neonates with rBCG-S1PT immunization, an efficient booster effect may be obtained by the whole-cell pertussis components in the normal DTP immunization schedule. Therefore, immunization with rBCG-S1PT has the potential to cover the immunological window at early life.

Finally, since BCG is already given at birth to infants in most developing countries, implementing the present recombinant vaccine would pose no significant alteration or cost burden in the current vaccination schedule. Thus, rBCG-S1PT may, in the future, substitute BCG as a combined vaccine against tuberculosis and pertussis. Therefore, our results bring a real prospect to protect newborns still susceptible to *B. pertussis* infection.

Acknowledgements

The experiments reported herein were performed according to principles set forth in the Guide for Care and Use of Laboratory Animals and approved by the Committee for Ethics in the Use of the Animals of Instituto Butantan. This work was supported by the Fundação de Apoio à Pesquisa do Estado de São Paulo- FAPESP (Ref. No 96/11539-0) and Fundação Butantan, São Paulo, SP – Brazil. I. P. Nascimento was supported by a FAPESP Post-Doctoral fellowship (Ref. No. 02/01748-4). We thank Dr. Eliane N. Miyaji for critical reading of the manuscript.

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