NOTES

Dose-Dependent Immune Response to *Mycobacterium bovis* BCG Vaccination in Neonates

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In 10-week-old infants vaccinated at birth with Japanese *Mycobacterium bovis* BCG, the number of dermal needle penetrations correlated positively with frequency of proliferating CD4+ T cells in whole blood following BCG stimulation for 6 days but did not correlate with secreted cytokine levels after 7 h or interferon CD4+ T-cell frequency after 12 h of BCG stimulation.

The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine is the only vaccine against tuberculosis (TB). Various BCG isolates, with well-characterized genetic differences, are currently used as commercial vaccines (1, 3, 9). In addition, different routes of BCG administration are used for vaccine delivery: i.e., intradermal injection or percutaneous puncture of the BCG-exposed skin by a multiprong device. The relative efficacies of the different strains of BCG and the different routes of vaccination are not clearly defined.

Protection against TB is dependent on T-cell-mediated immunity (4, 6–8, 12, 21, 24). The helper type 1 (Th1) CD4+ T cell producing interferon-gamma (IFN-γ) appears to be important for protection during primary infection with *Mycobacterium tuberculosis* and other mycobacteria (2, 5, 11, 18, 22). Similarly, following newborn and adult BCG vaccination, isolated peripheral blood mononuclear cells respond to ex vivo mycobacterial antigen stimulation by CD4+ T-cell proliferation and gamma interferon (IFN-γ) production. Therefore, these host responses have been widely used to measure antigen-specific memory T-cell immunity induced by BCG vaccination (16, 17, 20, 23).

Using two recently described short-term whole-blood assays and one 6-day T-cell proliferation assay to investigate BCG-induced T-cell immune responses in vaccinated neonates (10, 13, 14), we examined whether coincidental variation in the relative amounts of antigen introduced into the epidermis upon percutaneous administration of the Japanese BCG (JBCG) vaccine at birth correlates with any of the immune responses measured 10 weeks postvaccination.

Enumeration of the number of epidermal needle punctures following vaccination of neonates. Thirty-one infants vaccinated at birth with JBCG (Tokyo-172 BCG substrain, from Japan BCG Laboratory) administered percutaneously were recruited into the study at their routine 10-week visit to the primary health care clinic in the Western Cape Province of South Africa. The study protocol was approved by the Institutional Review Boards of the University of Cape Town and the Public Health Research Institute/University of Medicine and Dentistry of New Jersey, and their respective human experimentation guidelines were adhered to. Written informed consent was obtained from the infant’s parent or legal guardian. Human immunodeficiency virus-positive (by enzyme-linked immunosorbent assay) newborns and neonates exposed to a TB-positive index case during the first 10 weeks of life were excluded. The percutaneous immunization involved evenly spreading a drop containing 80 mg/ml JBCG (3 × 10^5 CFU/ml) over the skin followed by two superficial perforations of the epidermis with a nine-prong device (Japan BCG Laboratory) (Fig. 1A). The numbers of needle puncture scars visible on each infant’s forearm (Fig. 1B) 10 weeks after neonatal vaccination were graphically recorded (Fig. 1C, insert) and counted. Sixty-seven percent of infants (22 individuals) showed 18 clearly demarcated scars (the maximal number) (Fig. 1C). Thirty-three percent of infants (9 individuals) had fewer than 18 scars visible (but no less than 15). Thus, it may be presumed that in 33% of infants the dose of JBCG inoculated into the skin was less than optimal.

BCG-specific T-cell intracellular IFN-γ and soluble cytokine production (short-term stimulation assays). The frequency of mycobacterium-specific CD4+ T cells (as a percentage of total CD4+ cells) in peripheral blood of 10-week-old vaccinated infants was determined by quantifying IFN-γ+ CD4+ cells after short-term (12 h) ex vivo stimulation of whole blood with JBCG using flow cytometric detection of surface...
marker and intracellular cytokine (10, 14). Results were plotted against the number of vaccine scars visible on the arm for each infant (Fig. 1D). Correlation analysis indicated that the relative dose of percutaneously administered JBCG, as assessed by the number of vaccine needle scars, did not predict the frequency of IFN-γ CD4+ T cells (Fig. 1D and Table 1). A similar lack of correlation was observed when Danish BCG (DBCG) or purified protein derivative (PPD) was used for ex vivo stimulation of the whole blood to induce IFN-γ CD4+ T cells (Table 1). The frequency of IFN-γ CD8+ T cells induced by ex vivo exposure to JBCG, DBCG, or PPD also did not correlate with the number of scars. The levels of the six secreted cytokines and the IFN-γ/interleukin-4 (IL-4) cytokine ratio, measured by flow cytometric bead array kit (Th1/Th2 cytometric bead array; BD Biosciences) in the supernatants after 7 h of ex vivo stimulation of whole blood with JBCG or DBCG (10, 14), were also not significantly influenced by the dose of JBCG inoculated into the skin (Table 1).

**BCG-specific T-cell proliferation (6-day stimulation assay).** We next examined antigen-specific lymphoproliferation, using a novel whole-blood assay in which bromodeoxyuridine (BrdU) is incorporated into proliferating cells after 6 days of exposure to JBCG, DBCG, or PPD in culture. BrdU-positive cells were identified by flow cytometry (10). When this assay was used, the percentage of CD4+ T cells that proliferated in response to both JBCG and DBCG, as well as to PPD, correlated positively with the number of vaccine scars recorded (P < 0.05) (Fig. 1E and Table 1). The frequency of CD8+ T cells proliferating in response to ex vivo stimulation with JBCG, DBCG, or PPD did not correlate significantly with vaccine dose (Table 1). These results are supported by a previous study by Lowry et al. looking at the effect of four different doses of percutaneous BCG on the cellular immune response in adults (19).

Twelve-hour (short term) stimulation of blood allows quantification of vaccination-induced effector/effector memory T cells that produce IFN-γ and other cytokines, without in vitro expansion of the T cells (14). When this short-term assay was compared with the 6-day whole-blood proliferation assay, the two assays did not correlate (10). This suggested that the “immediate” (12 h) effector/effector memory responses, observed prior to any ex vivo proliferation taking place, were different from the 6-day proliferating, central memory CD4+ T-cell response. Thus, T cells primed to produce IFN-γ (as part of the immediate effector/effector memory response) after only a few hours of ex vivo mycobacterial stimulation may belong to a different subset of T cells from those primed to proliferate following longer ex vivo stimulation. It seems, therefore, that

![FIG. 1. (A) Multipuncture device used to administer the JBCG vaccine percutaneously. (B) Vaccination scars in a 10-week-old infant, vaccinated at birth with JBCG administered percutaneously. (C) Frequency histogram of the number of needle scars visible for each of the 31 study infants. (C, insert) Example of how the vaccination site seen in panel B was recorded for the study. (D) Comparison of JBCG percutaneous vaccination scar number and specific IFN-γ CD4+ T-cell frequency following ex vivo stimulation of whole blood with JBCG for 12 h. (E) Comparison of JBCG percutaneous vaccination scar number and frequency of proliferating BrdU+ CD4+ T cells following ex vivo stimulation of whole blood with JBCG for 6 days. The Spearman test was used to determine statistical significance of correlations for the data shown. A P value of 0.05 was considered significant.](image-url)
The short- and long-term assays measure different aspects of BCG-induced immunity. Similarly, Hoft et al. have reported that different immunologic assays measure unique aspects of mycobacterial immunity (15). This suggests that only one of the T-cell populations evaluated (i.e., the proliferating central memory CD4+ T cells) is sensitive to small changes in the dose of antigen delivered into the skin during vaccination.

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