Population Differences in Immune Responses to Bacille Calmette-Guérin Vaccination in Infancy

Maeve K. Lalor,1,a Anne Ben-Smith,1,4,a Patricia Gorak-Stolinska,1 Rosemary E. Weir,1 Sian Floyd,2 Rose Blitz,1 Hazzie Mvula,4 Melanie J. Newport,2 Keith Branson,2 Nuala McGrath,2 Amelia C. Crampin,2,4 Paul E. M. Fine,2 and Hazel M. Dockrell1

1Immunology Unit, Department of Infectious and Tropical Diseases, and 2Infectious Disease Epidemiology Unit, Department of Epidemiology and Public Health, London School of Hygiene and Tropical Medicine, London, and 3Department of Medicine, Brighton and Sussex Medical School, University of Sussex, Falmer, Brighton, United Kingdom; 4Karonga Prevention Study, Chilumba, Karonga District, Malawi

Bacille Calmette-Guérin (BCG) vaccination induces a marked increase in the interferon (IFN)–γ response to Mycobacterium tuberculosis purified protein derivative (Mtb PPD) in UK adolescents, but not in Malawian adolescents. We hypothesized that Mtb PPD-induced IFN-γ after BCG vaccination would be similar in infants from these 2 countries. Infants were vaccinated with BCG during the first 3–13 weeks of life. Three months after BCG vaccination, 51 (100%) of 51 UK infants had an IFN-γ response to Mtb PPD, compared to 41 (53%) of 78 of Malawian infants, in whom responses varied according to their season of birth. We conclude that population differences in immune responses after BCG vaccination are observed among infants, as well as among young adults.

Bacille Calmette-Guerin (BCG), a live attenuated strain of Mycobacterium bovis, is the only vaccine available against tuberculosis and has been part of the World Health Organization’s Expanded Program on Immunisation since 1974. Most countries, including Malawi, give BCG at or soon after birth [1]. A recent policy change in the United Kingdom has led to targeted vaccination of high-risk infants [2].

Clinical trials of BCG vaccination show variable efficacy (0%–80%) against pulmonary tuberculosis in adults, providing good protection against pulmonary tuberculosis in the United Kingdom, but little protection in Malawi [3]. BCG vaccination trials involving infants show consistently high efficacy against the severe forms of childhood tuberculosis [4].

We observed a large increase in interferon (IFN)–γ production in response to M. tuberculosis purified protein derivative (Mtb PPD) in BCG-vaccinated adolescents in the UK, but a relatively small mean change in IFN-γ production in Malawian adolescents who had an IFN-γ response to mycobacterial antigens prior to vaccination [5]. Differences in immune responses to BCG vaccination between the 2 populations may be the result of differing levels of prior exposure to environmental mycobacteria [6]. We expected Malawian and UK infants to have no or minimal prior mycobacterial exposure and therefore to have similar responses to BCG vaccination. We compared T cell responses induced by BCG vaccination of infants in Malawi and the United Kingdom by measuring IFN-γ released into supernatants from diluted blood cultures stimulated with Mtb PPD.

SUBJECTS, MATERIALS, AND METHODS

Recruitment and study design. A total of 117 UK infants were recruited at health centers after informed maternal consent was obtained. Infants who received BCG intradermally (n = 62) (Danish strain 1331, 0.05 mL; Statens Serum Institut) during the first 3–13 weeks of life
(median, 7 weeks) were recruited from Waltham Forest Primary Care Trust (PCT), and unvaccinated, age-matched infants (n = 55) were recruited from Redbridge PCT. Blood samples were obtained from vaccinated infants 3 months (i.e., 12–16 weeks; n = 51) and 12 months (i.e., 50–56 weeks; n = 38) after vaccination. Blood samples were obtained from unvaccinated, age-matched infants at approximately 6 (n = 36) and 15 months of age (n = 34).

In Malawi, recruitment took place at Chilumba Rural Hospital, Karonga. After obtaining informed consent and receiving counseling, women were tested for HIV infection and offered nevirapine if they tested positive. A total of 615 infants were vaccinated with BCG (Danish strain 1331, 0.05 mL; Statens Serum Institut); blood samples were obtained from 590 infants 3 months after BCG vaccination and from 552 infants 12 months after BCG vaccination. Infants were vaccinated at between 0 and 198 days of life; 383 (62%) of 615 were vaccinated during the first week of life. A subset of 109 Malawian infants vaccinated between 3 and 13 weeks of life were compared with the UK infants who were vaccinated at the same age. Infants who were born to HIV-positive mothers (8 [7%] of 109) were excluded.

Sample collection took place between March 2003 and November 2005. Approval for the study was given by the Redbridge and Waltham Forest Health Authority Local Research Ethics Committee, the ethics committee of the London School of Hygiene and Tropical Medicine, and the National Health Sciences Research Committee of Malawi.

Whole blood assay and IFN-γ ELISA. Whole blood assays and ELISA for IFN-γ were carried out as described elsewhere [5, 7]. Heparinized whole blood was diluted 1:9 and cultured on the day of collection with Mtb PPD (RT49, lot 204; Statens Serum Institut) at a concentration of 5 μg/mL. PHA-P (Difco Laboratories/Becton Dickinson; concentration, 5 μg/mL) was used as a positive control, and medium alone (RPMI 1640) was used as the negative control. Cultures were incubated at 37°C with 5% CO₂; supernatants were harvested on day 6 and stored at −70°C until assayed for IFN-γ in single 100-μL samples by quantitative ELISA (detection limit, 31 pg/mL).

Infants were grouped according to the season of their birth. For the UK infants, the seasons used were autumn (September–November), winter (December–February), spring (March–May), and summer (June–August). In Malawi, the seasons used were the warm and rainy season (January–May), the cool and dry season (June–September), and the hot and dry season (October–December).

Statistical analysis. Data were double entered and verified, and then analyzed by use of Stata (version 9; Stata). Negative control values were subtracted from all IFN-γ ELISA results. If the supernatant from diluted whole blood cultures stimulated with Mtb PPD for 6 days contained >62 pg/mL IFN-γ, twice the limit of detection of the IFN-γ ELISA, the infant was considered to have a positive IFN-γ response to Mtb PPD [8]. The proportion of infants who had a positive IFN-γ response was compared across groups by use of χ² tests. Nonparametric Mann-Whitney tests were used to compare IFN-γ responses among infants who had a positive response.

RESULTS

IFN-γ responses to Mtb PPD. In the United Kingdom, all (51 of 51) vaccinated infants had a positive IFN-γ response to Mtb PPD 3 months after vaccination, and 36 (95%) of 38 had a positive response 12 months after vaccination. Unvaccinated control subjects had no IFN-γ response to Mtb PPD at either time point (0 of 36 and 0 of 34 infants, respectively), (P < .001 for comparison of vaccinated and unvaccinated infants at either time point). In Malawi, only 41 (53%) of 78 vaccinated infants had a positive response at 3 months, and 37 (48%) of 77 had a positive response at 12 months. The median responses at 3 months in vaccinated infants who had a positive response were 1779 pg/mL (UK infants) and 289 pg/mL (Malawian infants); there was a decline in the median response by the 12-month time point to 926 pg/mL and 204 pg/mL, respectively (figure 1) (P < .001 for both 3 and 12 months). In Malawi, 17 (28%) of 61 infants did not have a response at either time point, 16 (26%) had a positive response only at 3 months, 12 (20%) had a positive response only at 12 months, and 16 (26%) had a positive response at both time points (only 61 infants had blood samples taken at both 3 and 12 months). IFN-γ responses to Mtb PPD in the Malawian infants who were vaccinated within the first week of life were similar: 135 (64%) of 210 and 114 (43%) of 267 had a positive response at 3 and 12 months, respectively. We found no technical factor to explain the differences between the UK and Malawian results (see the appendix, which appears only in the electronic version of the Journal).

IFN-γ responses to positive and negative control cultures. Positive responses to unstimulated control cultures were rare (<1% [1 of 159] in UK infants and <8% [11 of 155] in Malawian infants). Among vaccinated UK infants, positive IFN-γ responses to the mitogen phytohemagglutinin (PHA) were seen in 45 (88%) of 51 infants 3 months after BCG vaccination and in 28 of (74%) 38 infants 12 months after vaccination. In unvaccinated control subjects, positive responses were seen in 27 (75%) of 36 infants 3 months after vaccination, but in only 9 (27%) of 34 infants 12 months after vaccination. In Malawi, positive IFN-γ responses to PHA were seen in 40 (51%) of 78 infants 3 months after vaccination and in 35 (45%) of 77 infants 12 months after vaccination. Among the Malawian infants, there was an association between a positive IFN-γ response to Mtb PPD and a positive response to PHA 3 months after BCG vaccination; 23 (62%) of the 38 infants who did not have a response to PHA also did not have a response to Mtb PPD, whereas 14 (38%) of the 40 infants who responded to PHA did not respond to Mtb PPD (P = .024). This trend was also observed 12 months after BCG vaccination (P = .055). When the analysis was restricted to the infants who had a response to PHA, the
Figure 1. Interferon (IFN)-γ responses to Mycobacterium tuberculosis purified protein derivative (Mtb PPD). A, Histograms showing IFN-γ responses to Mtb PPD in unvaccinated UK infants, vaccinated UK infants, and vaccinated Malawian infants 3 and 12 months after Bacille Calmette-Guérin (BCG) vaccination. Proportion of responses (proportion). B, Box and whisker plots showing IFN-γ responses in vaccinated UK and Malawian infants 3 and 12 months after BCG vaccination. Plots show median values and interquartile range (box), the upper and lower adjacent values (whiskers), and outliers (circles).
proportion of infants who responded to *Mtb* PPD in Malawi was much lower than the proportion in the UK (26 [63%] of 41 and 21 [57%] of 37 Malawian infants had a response at 3 and 12 months, respectively, whereas 45 [100%] of 45 and 27 [96%] of 28 UK infants had a response at 3 and 12 months, respectively) \( (P/0.001) \).

**Skin test response and scar size.** Mantoux skin tests were performed for Malawian infants 3 months after BCG vaccination. Of 76 infants, 37 (49%) had no skin test response (0 mm), whereas 39 (51%) had a skin test response. Of these infants, 20 (26%) had a response between 1 and 9 mm, and 19 (25%) had a response >10 mm. There was a strong association between skin test response and IFN-\( \gamma \) measured in supernatants stimulated with *Mtb* PPD \( (P < .001) \). The median BCG vaccination scar size 3 months after BCG vaccination was greater among the UK infants (5 mm) than among the Malawian infants (3 mm) \( (P < .001) \), with 51 (100%) of 51 UK infants forming a scar while only 42 (79%) of 53 Malawian infants formed a scar (scar data was not available for all infants).

**Seasonal variation in responses.** In Malawi, infants who were born during the hot and dry season were more likely to have a positive IFN-\( \gamma \) response (>62 pg/mL) to *Mtb* PPD 3 months after BCG vaccination (10 [71%] of 14) than were infants born during the cool and dry season (12 [55%] of 22) who were in turn more likely to have a positive response than were infants born during the warm and rainy season (19 [45%] of 42) \( (P = .230) \) (figure 2A). More infants born during the hot and dry season had a high response (>500 pg/mL), compared with infants born during the other seasons \( (P = .002) \) (figure 2A). The median IFN-\( \gamma \) response to *Mtb* PPD among those who had a positive response also varied by season (206 pg/mL during the warm and rainy season, 240 pg/mL during the cool and dry season, and 1729 pg and mL during the hot and dry season) \( (P < .001) \).

As the number of Malawian infants in this restricted data set was small, the full data set (615 infants) was also analyzed. Three months after vaccination, the odds of having a positive IFN-\( \gamma \) response were higher for infants born during the hot and dry season and those born during the cool and dry season, compared...
with those born during the warm and rainy season (odds ratios [ORs], 5.1 [\(P < .001\)] and 1.8 [\(P = .04\)], respectively). Twelve months after vaccination, the trend was in the same direction, although weaker (ORs, 2.1 [\(P = .005\)] and 1.3 [\(P = .28\)], respectively). The trend was similar when we used a high cutoff for an IFN-\(\gamma\) response (\(>500\) pg/mL), and it was seen during each of the 2 years of the study (data not shown). Analysis of IFN-\(\gamma\) responses to \(Mtb\) PPD according to season of BCG vaccination instead of season of birth gave similar results.

The median IFN-\(\gamma\) response among UK infants was higher among those born during the spring (2078 pg/mL) and summer (1922 pg/mL), compared with those born during the autumn (1342 pg/mL) and winter (1196 pg/mL) (figure 2) (\(P = .04\)).

**Birth weights.** Only 7% of Malawian infants were considered to have low birth weight, and birth weight did not confound the association between birth season and positive IFN-\(\gamma\) response. In Malawi, there was a weak association between birth weight and positive IFN-\(\gamma\) response to \(Mtb\) PPD at 3 months (\(P = .116\)), but not at 12 months (\(P = .6\)).

**DISCUSSION**

In this study, infants responded differently to BCG vaccination in the United Kingdom and Malawi. Whereas all vaccinated infants in the UK group (51 of 51) had a strong IFN-\(\gamma\) response to \(Mtb\) PPD 3 months after BCG vaccination, only 41 (53%) of 78 infants in Malawi had a positive IFN-\(\gamma\) response at this time point, and responses were of a lower magnitude than those observed in UK infants. The proportion of infants who had a positive IFN-\(\gamma\) response to \(Mtb\) PPD 3 months after BCG vaccination was maintained up to the 12-month time point in both the United Kingdom and Malawi, whereas the median response decreased in the United Kingdom but not in Malawi. There was greater variability in response among Malawian infants, with 28 (46%) of 61 infants having a positive response at only 1 time point. In Malawi, 3 months after BCG vaccination only 36 (47%) of the infants had a skin test response \(>5\) mm, although this percentage was higher than that seen 2 months after BCG vaccination in Guinea Bissau (in that study, 39% of subjects had a response \(>1\) mm) [9]. Scars were present 3 months after BCG vaccination for only 42 (79%) of 53 Malawian infants and were smaller than the median size of scar observed among UK infants. Unvaccinated control subjects in the United Kingdom did not produce any detectable IFN-\(\gamma\) response to \(Mtb\) PPD at either time point.

The differences observed in the responses to BCG vaccination in UK and Malawian adolescents have been attributed to the high proportion of Malawian individuals already exposed and sensitized to a variety of environmental mycobacterial antigens prior to vaccination [6]. Masking or blocking of the BCG response through exposure to environmental mycobacteria remains a plausible explanation, although we were unable to demonstrate a relationship between prior IFN-\(\gamma\) responses to specific environmental mycobacterial antigens and BCG-attributable changes in IFN-\(\gamma\) response to \(Mtb\) PPD in UK or Malawian adolescents [10]. The differences observed between UK and Malawian infants are unlikely to be the result of prior environmental exposure, as Malawian infants vaccinated during the first week of life had low IFN-\(\gamma\) responses to \(Mtb\) PPD.

There has been no formal evaluation of the neonatal protection offered by BCG vaccination against childhood forms of tuberculosis in Malawi. Given the large population differences observed in the immune response to BCG vaccination among UK and Malawian infants, it is possible that BCG vaccination does not offer equal protection to infants in different countries. Studies of neonatal BCG vaccination in some other African countries, such as South Africa and The Gambia, have shown strong induced IFN-\(\gamma\) responses in similar assays. In The Gambia, IFN-\(\gamma\) responses to \(Mtb\) PPD measured in PBMC cultures were similar to adult responses [11]. In South Africa, IFN-\(\gamma\) was measured by flow cytometry after stimulation of whole blood with live BCG 10 weeks after vaccination [12]. However, a randomized trial in Guinea Bissau that examined the effect of vitamin A supplementation on immune responses to BCG vaccination, which used a whole blood assay similar to the one we used, found a comparably low median IFN-\(\gamma\) response (199 pg/mL to \(Mtb\) PPD), similar to the median response among Malawian infants in our study [9].

IFN-\(\gamma\) responses to PHA were lower than expected at 3 and 12 months after BCG vaccination among both UK and Malawian infants. In the UK, although 51 (100%) of 51 vaccinated infants had a positive response to \(Mtb\) PPD 3 months after BCG vaccination, only 45 (88%) of 51 infants had a positive response to PHA. In Malawi, positive responses to PHA were low, with only 40 (51%) of 78 infants responding 3 months after BCG vaccination. In future studies, staphylococcal enterotoxin B [12] may be a better positive control to use for infants than 5 \(\mu\)g/mL PHA.

IFN-\(\gamma\) responses to \(Mtb\) PPD 3 months after BCG vaccination varied according to the season of birth of the infant. In Malawi, the proportion of infants who had a positive IFN-\(\gamma\) response was higher for infants born during the hot and dry season, compared with those born during the other 2 seasons, and the median response among those who had a positive response was similar to that observed among UK infants. In the United Kingdom, a difference in IFN-\(\gamma\) response to \(Mtb\) PPD according to season of birth was also observed, with greater responses observed in infants born during the winter, compared with those born during the other 2 seasons, and the median response among those who had a positive response was similar to that observed among UK infants. In the United Kingdom, a difference in IFN-\(\gamma\) response to \(Mtb\) PPD according to season of birth was also observed, with greater responses observed in infants born during the summer. Seasonal variation in responses to rabies and typhoid vaccinations have been reported in The Gambia and Pakistan [13], and a recent study of BCG-vaccinated infants in The Gambia found higher proportions of CD4 T cells expressing CD154 at 12 months of age in infants born during the wet season, compared with those born during the dry season [14]. Our observed seasonal variation in IFN-\(\gamma\) responses to BCG vaccination suggest that environmental factors play a role in vaccine induced immunity. These could in-
include seasonal differences in the burdens of infections, such as malaria or respiratory infections during the wet season in Malawi and common respiratory infections during the winter in the UK. The varying responses in Malawi could also be the result of nutritional factors, which vary by season, depending on the availability of food.

Other factors that may also be influencing the different responses seen in Malawian and UK infants include differences in the maturation of the immune system, T cell clonal responses [15], the burdens of maternal infectious diseases, or the schedules of other vaccinations. There was no association between lymphocyte counts and the IFN-γ response to Mtb PPD (results not shown), but more detailed analyses of the relevant subpopulations of T cells, regulatory cells, and antigen-presenting cells is warranted.

To our knowledge, this is the first study showing that population differences in response to BCG vaccination occur in infants as well as adults, by measuring secretion of IFN-γ in response to Mtb PPD at 3 and 12 months after vaccination. Although IFN-γ production by itself does not provide a correlate of protection, much evidence suggests that it contributes to immunity and that it provides a good indicator of vaccine immunogenicity. This finding has important implications for the use of BCG and the development and testing of future tuberculosis vaccines designed to boost the immune responses induced by BCG. It may also have implications for immune responses to other infant vaccinations and warrants further study.

Acknowledgments

We would like to acknowledge the excellent technical assistance of Mr. Kandakoune Makamo. We would also like to acknowledge Dr. Christine Sloczynska at Waltham Forest Primary Care Trust and Dr. Makki Hameed at Redbridge Primary Care Trust and Shakuntala Patel for their help with the UK infant study.

References

APPENDIX

SUPPLEMENTARY MATERIAL ON QUALITY CONTROL

BCG vaccine source, transport, storage, delivery. The same strain of BCG (Danish 1331) from Statens Serum Institut in Copenhagen was used in both the United Kingdom and Malawi. There were no known problems with the cold chain at any point, from time of purchase through storage to time of delivery. BCG was given intradermally by trained staff in both locations. As 11 (21%) of 53 Malawian infants did not develop a scar, one could argue that the vaccine was not successfully administered, but restricting analysis to infants who developed a scar did not alter the results.

Blood sample storage, transport, and processing times. Blood was collected and transported to the lab to be processed. The time it took to get the blood into culture was similar in both countries, and a delay in processing did not affect the IFN-γ response to antigen.

Blood culture methods, timing, reagents, operators, and equipment. Antigen plates were prepared in advance and thawed as needed. Antigen plates were, on average, frozen for longer periods in the United Kingdom, but plates frozen for longer periods did not yield lower IFN-γ responses to Mtb PPD or PHA (data not shown). Reagents and methods were identical, equipment was equivalent, and staff were exchanged between laboratories. To check whether there was a period when a piece of equipment or a procedure had not worked, data were analyzed according to month of blood collection, but infants who had an IFN-γ response to Mtb PPD were found in all periods.

Supernatant storage. Supernatants were stored in the −80°C freezer until the ELISA were performed. Samples were only thawed once, prior to ELISA testing, and supernatants tested ≤3 years later had IFN-γ responses similar to those obtained during initial testing (data not shown).

ELISA method, reagents, quality control, and operators. The same standard operating procedure was used for the ELISA in both countries. The reagents and equipment were the same at each site. To control for interplate and intraplate variation, a positive control supernatant was prepared at each site, and was tested in duplicate on each ELISA plate. The coefficient of variation between plates in the United Kingdom (n = 16) was 7%, with a mean value of 790 pg/mL. In Malawi, 2 positive controls were used; the first gave a mean value of 673 pg/mL (n = 63) and the coefficient of variation was 9.7%, and the second gave a mean value of 976 pg/mL (n = 27) with a coefficient of variation of 10.7%. ELISA plates that had a positive control value that differed by >20% from the mean of all plates were repeated. ELISA plates that did not have a satisfactory standard curve were repeated. The data included in the analysis presented here are from ELISA plates that passed all quality controls.

At various times in the study, the UK and Malawian positive control supernatants were exchanged between the 2 sites, evaluated according to the laboratories’ routine procedure, and found to be comparable. The differences among operators and among time periods when the ELISA plates were tested were relatively small. To further control for quality between the 2 sites, after the study was completed 20 control supernatants were prepared over the range of the standard curve and tested at both sites. Overall, there was no evidence of a difference between the 2 sites; in particular, very similar values for the lower-value controls were obtained, and thus the level of detection was similar at the 2 sites (P = .54)