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Monocyte Antimycobacterial Activity before and after *Mycobacterium bovis* BCG Vaccination in Chingleput, India, and London, United Kingdom

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Monocytes from purified protein derivative S Mantoux-negative children and young adults inhibited intracellular growth of *Mycobacterium microti* more in Chingleput than in London. *Mycobacterium bovis* BCG vaccination did not enhance bacteriostasis with the Indians but did so with the Londoners. No evidence was found for involvement of cytokines such as macrophage-activating factor and granulocyte macrophage colony-stimulating factor in the differences.

The failure of *Mycobacterium bovis* BCG vaccination to confer protection against tuberculosis in some parts of the world and not others is a cause of great concern but is without adequate explanation (8). We developed a test which revealed an increased antimycobacterial activity of peripheral blood monocytes after BCG vaccination in British school children (1), in whom BCG vaccination has high protective efficacy (15). We have now applied the test before and after vaccination in Chingleput, in southern India, where BCG vaccination gives little or no protection against tuberculosis (16, 17), and compared results with those from British children. We also sought a correlation between monocyte antimycobacterial activity and cytokine release from stimulated peripheral blood leukocytes.

The Indian subjects (10 male and 8 female) were selected from villages in the Chingleput district of Tamil Nadu adjacent to where the major BCG vaccine trial was carried out (17). Selection was initially on the basis of age and a Mantoux skin test considered negative by the criteria of the vaccine trials (see below). The subjects' ages ranged from 12 to 25 years (mean, 14.6). The British vaccinees (8 male and 3 female) were undergoing routine BCG vaccination under the Schools Medical Service. Their ages ranged from 13 to 15 years (mean, 14.3). Clearance by local medical ethics committees and informed consent were obtained. All subjects met stringent criteria of eligibility, which included good health; normal chest radiographs; and no history of tuberculosis or other chronic diseases, of antituberculosis chemotherapy, or of BCG vaccination. All were Mantoux skin tested with 3 or 4 U of purified protein derivative S (PPD-S) and 10 U of PPD-B (Statens Seruminstitut, Copenhagen, Denmark), and all were Mantoux negative to PPD-S (72-h reaction diameters, 0 to 7 mm to 3 U in Chingleput [17] and 0 to 3 mm to 4 U in London). Immediately before vaccination, a blood sample was taken into citrate anticoagulant (CDP Adenine 1; Travenol Laboratories, Thetford, England). Vaccination was by intradermal injection of 0.1 μg of the freeze-dried Danish BCG strain 1331 (Statens Seruminstitut) of the batch used in the vaccine trial. Eight weeks after vaccination, blood was again taken and the PPD-S skin test was repeated. Blood samples were transported to arrive in the laboratory within 2 to 3 h. A sample from a blood bank donor (Madras) or an adult laboratory volunteer with a known history of previous BCG vaccination (London) was processed in parallel as a control.

The antimycobacterial assay was detailed previously (1) and used *Mycobacterium microti* as the target because of technical difficulties in quantifying infection with virulent *Mycobacterium tuberculosis* in monocytes. *M. microti* has a limited capacity to multiply in humans and is an effective live vaccine against tuberculosis (11). In brief, a mononuclear cell suspension from Ficoll-Hypaque was split into two parts in RPMI 1640 medium containing 5% autologous serum. One part was incubated (37°C, 5% CO_2) with dead (γ-irradiated) *M. tuberculosis* H37Rv (one bacterium per three mononuclear cells) to provide antigen-stimulated lymphocytes. The other part was infected with *M. microti* in microtiter tray wells at a low bacterium/monocyte ratio (1:400; 5×10^2 bacteria and 2×10^5 mononuclear cells per well), and after 24 h of incubation to allow phagocytosis and monolayer development, followed by removal of nonattached bacteria, the monolayers were pulsed with stimulated lymphocytes for 2 h every day for 3 days. Parallel wells had medium changes without lymphocyte exposure. A series of four sequential daily estimates of the rate of multiplication of the bacteria remaining in the monolayers was obtained from CFU counts of *M. microti* in representative monolayers and in their discarded supernatants and rinse solutions. Control wells contained medium and bacteria only. No antibiotics were present in culture media at any time.

Most blood samples were processed and tested for antimycobacterial function by one person (S.H.C.) in both Madras and London to ensure technical compatibility between centers. Growth rates were variable during the first 2 days in monocytes as the monolayers became established, and analysis was confined to data from days 3 and 4. Analysis of variance of $\log_{10}$ CFU (microcomputer software, INSTAT [Statistical Services Centre, University of Reading, Reading, United Kingdom] and GENVAR [Applied Statistics Re-

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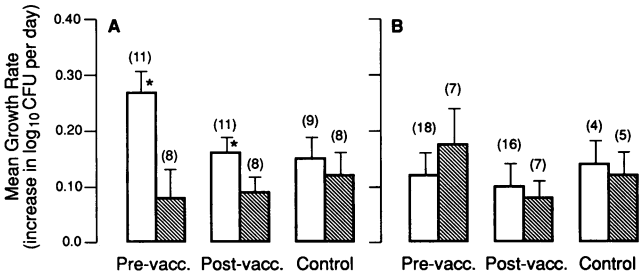

FIG. 1. Mean growth rates of *M. microti* in monocytes from British (A) and Indian (B) subjects. Monocytes were obtained from the same initially Mantoux-negative individuals before BCG vaccination (pre-vacc.) and 8 weeks after (post-vacc.) and tested alongside monocytes from Mantoux-positive adults (control). Culture wells contained either monocyte monolayers (open bars) or culture medium only (hatched bars). The number of subjects tested is shown in parentheses. *, significant difference between growth rates ($P < 0.05$); error bars, standard error of the mean.

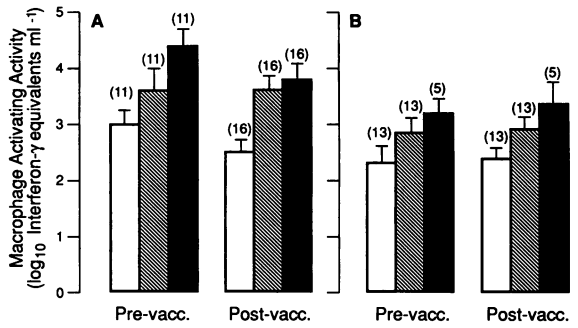

FIG. 2. Macrophage-activating factor activity in mononuclear cell supernatants from British (A) and Indian (B) subjects before (pre-vacc.) and 8 weeks after (post-vacc.) vaccination. Cells were incubated without added stimulus (open bars), with dead *M. tuberculosis* organisms (hatched bars), or with phytohemagglutinin (solid bars). Activity in cell supernatants was assayed as the capacity to enhance release of hydrogen peroxide from U937 cells in response to *M. tuberculosis* as a phagocytic stimulus. The number of subjects tested is shown in parentheses. Error bars, standard error of the mean.

search Unit, University of Kent, Canterbury, United Kingdom]) showed no significant differences between growth rates during days 3 and 4 or between lymphocyte-pulsed and nonpulsed wells, and the pooled data are shown in Fig. 1. Vaccination decreased the mean growth rate ($\log_{10}$ CFU/day) with British vaccinees (0.27 prevaccination versus 0.16 postvaccination; $P = 0.025$) while having no effect with Indian vaccinees. Growth rates were higher overall in monolayers from British vaccinees than from Indian vaccinees (0.22 versus 0.11, respectively; $P < 0.01$).

Decreased growth of *M. microti* in monocytes after vaccination of British subjects but not of Indian subjects suggests that this test may indeed reflect vaccine efficacy. It also adds to the limited direct evidence that in humans, as in animals, immunity against tuberculosis is likely to be mediated by direct action of mononuclear phagocytes (2, 6, 7, 14). Although host genetic factors (3) can not be ruled out, the findings are consistent with the hypothesis that BCG vaccination fails to protect people in southern India because the population is already maximally immunized by contact with environmental mycobacteria (12). This hypothesis more readily accommodates the twin observations of (i) the prevaccination difference between growth rates in British and Indian monocytes and (ii) the lack of effect of vaccination on growth rate in Indian monocytes in contrast to British monocytes. Our tests with PPD-B confirmed that the South Indian vaccinees had a greater sensitization to environmental mycobacteria of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* group than the British vaccinees (4). Furthermore, a 15-year follow-up of the BCG vaccination trial in Chingleput revealed a modest protective effect (17%) only in the youngest age group of vaccinees (1 to 14 years), comprising those least sensitized to environmental mycobacteria (16).

Several studies have indicated a capacity of cytokines to modulate human macrophage action against tubercle bacilli in vitro (5, 13). Although vaccination consistently increased the lymphocyte proliferative response to γ-irradiated *M. tuberculosis* (data not shown), vaccination had no effect on cytokine release with either British or Indian subjects. Cytokines were obtained from mononuclear cells incubated for 2 or 3 days with γ-irradiated *M. tuberculosis* (cell/bacterium ratio, 3:1), with phytohemagglutinin (0.5 μg ml^{-1}), or without added stimulus. Macrophage-activating factor activity in the supernatants was assayed as enhancement of U937 human monocyte-like cell line release of hydrogen peroxide in response to γ-irradiated *M. tuberculosis* (bacterium/U937 ratio, 50:1) (10) and expressed as gamma interferon equivalents by reference to recombinant human gamma interferon (Genentech Inc., donated by Boehringer-Ingelheim, Vienna, Austria). Results for 2- and 3-day cytokines did not differ significantly; pooled results are shown in Fig. 2. Preliminary results of assaying granulocyte macrophage colony-stimulating factor and interleukin-1β by enzyme-linked immunosorbent assay (INSIGHT; Media Resources Ltd., Darlinghurst, New South Wales, Australia) and tumor necrosis factor bioassay (9) have similarly failed to show significant effects of vaccination or differences between British and Indian subjects (not shown). In future, analysis of the earliest responses of defined lymphocyte subsets to monocytes presenting individual antigenic components might well resolve the relevance of differences in exposure to environmental mycobacteria.

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