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Demonstration of increased anti-mycobacterial activity in peripheral blood monocytes after BCG vaccination in British school children

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SUMMARY

A blood sample was taken from children aged 13–15 years immediately before BCG vaccination and 8 weeks after. The children were tuberculin skin-test negative to PPD-S before vaccination and positive after. Mononuclear cells were separated from the blood, infected with *Mycobacterium microti* at a low bacterium/monocyte ratio and allowed to form monolayers in microtitre wells. The infected monolayers were rinsed daily and the change in number of live bacteria in monolayers and supernatants was monitored by colony counts on agar. The cells were bacteriostatic during the first day, thereafter growth accelerated in pre-vaccination monolayers. When monolayers received pulsed exposures to autologous lymphocytes that had been incubated with whole dead tubercle bacilli the growth rates of *M. microti* were increased. However, growth rates in lymphocyte-pulsed monolayers were significantly lower after vaccination than before. It is proposed that this difference reflects the protective effect of vaccination.

Keywords Macrophages mycobacteria BCG vaccination

INTRODUCTION

BCG vaccination has been found to confer widely different degrees of protection against tuberculosis in different human populations throughout the world; for example contrast the 78% protection in British adolescents (MRC, 1963) and practically zero protection in South Indian subjects (Tuberculosis Prevention Trial, 1979). As the first stage of an investigation of possible immunological mechanisms underlying such differences we sought to develop a test capable of revealing acquired anti-tuberculosis immunity in human peripheral blood.

It is generally believed that acquired immunity against *Mycobacterium tuberculosis* is expressed by lymphocyte-activated macrophages killing or inhibiting the growth of the bacteria. Direct evidence for this has come from studies with mouse cells *in vitro* (Cahall & Youmans, 1975; Walker & Lowrie, 1981; Rook *et al.*, 1985; Khor, Lowrie & Mitchison, 1986; Flesch & Kaufmann, 1987) but it has proved difficult to obtain comparable evidence with human cells (Godal, Rees & Lamvik, 1971; Douvas *et al.*, 1985; Rook *et al.*, 1986; Steele *et al.*, 1986). This has left uncertainty over whether, or to what extent, either death or stasis in macrophages contributes to tuberculosis immunity in man (Lowrie & Andrew, 1988). The difficulty is largely a technical one, arising from the frequent failure of human mononuclear phagocytes to form monolayers as robust as those from mice. This means that conventional methods of assessing antibacterial action are inadequate, particularly since the kinetics are likely to be slow with a slow-growing organism like *M. tuberculosis*. A secondary difficulty is the variability between individuals in their background immunity, whether innate or acquired, which may mask specifically acquired immunity. Since BCG vaccination still confers protection against pulmonary tuberculosis in over 70% of vaccinees in the UK (Sutherland & Springett, 1987), we reasoned that a suitable test of anti-mycobacterial activity ought to detect the increase in immunity in such individuals if we made paired comparisons of peripheral blood cells before and after vaccination. We describe here a test we have developed which appears to detect the enhancement of immunity resulting from BCG vaccination. In this test freshly harvested peripheral blood monocytes are infected with *Mycobacterium microti* and subsequent bacterial growth is monitored by colony counts on agar.

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MATERIALS AND METHODS

Clinical protocol

Samples of peripheral blood were taken from school-children attending for routine BCG vaccination under the Schools

Medical Service. Clearance by the local Medical Ethics committees and parental consent were obtained. The children were aged 13–15 years, in good health, with no history of tuberculosis, anti-tuberculous chemotherapy or other chronic disease and with no previous BCG vaccination. Chest radiographs were normal. The children were Mantoux skin-test negative (72 h reaction diameter < 4 mm) to both 4 IU PPD-S and 10 IU PPD-B (Statens Serum Institut, Copenhagen, Denmark). Immediately before vaccination 45 ml of blood was taken into a syringe containing 6 ml of citrate anticoagulant (CPD Adenine 1; Travenol Labs., Thetford, England). Vaccination was by intradermal injection of 0·1 μg of the freeze-dried Danish BCG strain (Statens Serum Institut). Eight weeks after vaccination a further 45 ml of blood was taken and the skin-test with PPD-S was repeated and found to be positive (4 mm or more). Chest radiographs were again normal. Either two or three children were bled at a time and a 45 ml blood sample was always taken from one of several adult laboratory volunteers (with a known history of previous BCG vaccination) and processed in parallel as a control.

Mononuclear cells

Mononuclear cells and plasma were separated from citrated blood at ambient temperature (22°C) by centrifugation over Ficoll-Hypaque (specific gravity 1·085). Serum was collected after clotting the plasma with thrombin (700 NIH units/10 ml; Leo Pharmaceutical Products, Denmark). The cells were rinsed once with RPMI 1640 medium (Gibco, Paisley, Scotland), once with RPMI 1640 containing 5% (v/v) autologous serum and finally suspended to a concentration of 5×10^6/ml in RPMI 1640 without serum. Phase-contrast microscopy showed mononuclear cell yields to be $3{\cdot}6–7{\cdot}6 \times 10^7$ per 45 ml with > 95% viability judged by trypan blue exclusion.

Mycobacteria

M. microti OV254 was grown as a logarithmic phase culture in double strength Dubos Broth Base (Difco Laboratories, West Molesey, Surrey), rinsed twice in Hanks' balanced salt solution and briefly ultrasonicated to break up clumps (Khor, Lowrie & Mitchison, 1986). Prolonged ultrasonication (> 1 min) did not decrease bacterial viability. The infective inoculum for monolayers was prepared in RPMI 1640 medium to give a bacteria to leucocyte ratio of 1:200. *M. tuberculosis* H37Rv (TMC 102) for use as antigen was grown as a surface pellicle on Sauton's liquid medium (Difco), killed by gamma-irradiation (2·5 Mrad) and stored at −70°C. This stock was diluted for use in RPMI 1640 to give a final bacterium to leucocyte ratio of 1:3·3.

Assay of anti-mycobacterial activity

We found that blood from children yielded monolayers that were even more inclined to lose cells during culture than were monolayers from adults. We therefore developed an assay which both minimized and allowed for this cell loss. In outline the procedure was to first split the leucocyte preparation into two parts. One part was incubated with whole dead tubercle bacilli to provide antigen stimulated lymphocytes. The other part was infected with *M. microti* in microtitre tray wells at a low bacterium to monocyte ratio and, after 24 h incubation to allow phagocytosis and monolayer development, followed by removal of non-attached bacteria, the monolayers were pulsed with stimulated lymphocytes for 2 h every day for 3 days. The fate of the bacteria in the wells was monitored daily by determining the number of colony-forming units (CFU) of *M. microti* in representative monolayers and in their discarded supernatants and rinse solutions. This yielded a series of four sequential estimates of rate of multiplication of the bacteria remaining in the wells during a 24 h period.

In detail, wells for producing stimulated lymphocytes received 200 μl of leucocyte suspension (1×10^6 cells), 10 μl of antigen (3×10^5 *M. tuberculosis*) and 20 μl of autologous serum. Wells for infected monolayers received 200 μl of leucocyte suspension and 10 μl of logarithmic phase *M. microti* suspension (5×10^2 bacteria). Incubation was at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h the infected monolayers were gently agitated, the medium containing unattached leucocytes and bacteria was removed and the monolayers were gently rinsed with 200 μl RPMI 1640. Spent medium and rinse solutions were pooled for bacterial CFU counts. Fresh medium (200 μl RPMI 1640 containing 5% autologous serum) was then added and incubation continued. At 46, 70 and 94 h after infection 100 μl of culture supernatant was removed, without disturbing the cells at the bottom of the wells, and replaced by 100 μl of cells from the 'stimulated lymphocyte' wells that had been resuspended by gentle pipetting up and down. After incubation for 2 h the non-adherent cells were removed by gently pipetting up and down and removing the suspension. Fresh medium (200 μl) was then added. From a pair of wells at each time point the culture supernatant and non-adherent cell suspension that had been removed were kept for bacterial 'non-adherent' CFU counts. From those wells the monolayers were harvested by scraping the cells off with polytetrafluorethylene-tipped rods into their culture medium, repeating the process with a further 200 μl of RPMI 1640. The resulting pooled suspensions comprised the 'adherent' viable counts. The various suspensions for bacterial counts were briefly ultrasonicated to disrupt any cell clumps, then serially diluted 10-fold, first into 0·8 mg/ml digitonin to lyse monocytes then continuing in distilled water. Volumes of 50 μl were pipetted onto one-third segments of Dubos agar plates containing oleic acid, dextrose and catalase (Dubos agar + OADC supplement, Difco). The plates were incubated for 4 weeks at 37°C before colonies were counted.

Sham-pulsed monolayers were set up simultaneously and handled identically to the lymphocyte-pulsed monolayers except that pulsing was with RPMI 1640 containing 5% autologous serum only.

Bacteria-only wells were also established simultaneously and contained 5×10^3 *M. microti* in 200 μl RPMI 1640 containing 5% autologous serum. Every 24 h 100 μl of medium was removed without disturbing the sedimented bacteria and replaced with fresh medium. From pairs of wells the medium that had been removed was kept for bacterial CFU counts and the wells were scraped and harvested for CFU counts exactly as with monolayer wells.

Calculation of growth rates

Bacterial growth rates during each 24 h period were calculated as:

$$R = \mathrm{Log}_{10}(\mathrm{Adh}^{T24} + \mathrm{NonAdh}^{T24}) - \mathrm{Log}_{10}(\mathrm{Adh}^{T})$$

Where Adh^{T24} = adherent CFU at time T + 24 h, NonAdh^{T24} = non-adherent CFU at time T + 24 h, Adh^{T} = adherent CFU at

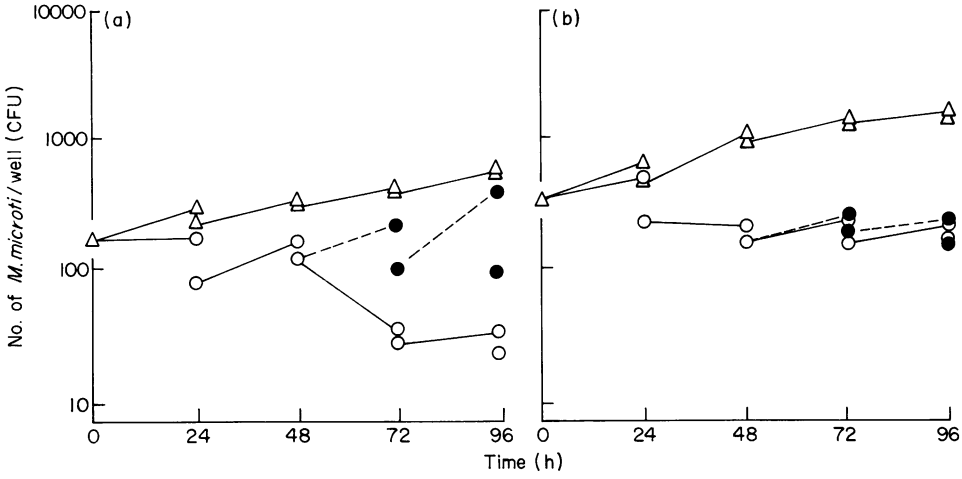

Fig. 1. Changes in number of viable *M. microti* after infection of macrophage monolayers obtained from a child before vaccination (a) and 8 weeks after vaccination (b). Mononuclear cells and bacteria were added to microtitre tray wells at time 0 and 24 h later the numbers of bacteria present were determined before and after rinsing the monolayers. At 48, 72 and 96 hours the bacterial numbers were determined before and after the monolayers had been pulsed either with autologous antigen-stimulated lymphocytes (●) or with medium only (○) and then rinsed. The pairs of identical symbols shown at each time represent the bacteria present in the wells before the rinsing and pulsing procedures (higher symbol) and afterwards (lower symbol). The connected data points thus represent the growth during the ensuing 24 h of the bacteria that remained monolayer-associated after rinsing. Control wells (△) contained bacteria in tissue culture medium only, 50% of which was replenished every 24 h.

time T. In the case of the first 24 h period, Adh is the CFU added to the well at time zero.

Statistical analysis. Counts of bacteria were $\log_{10}$ transformed and compared either by using Student's *t*-test for paired and unpaired samples or by analysis of variance. The relationship between the percentage of bacteria that were non-adherent and growth rate was investigated by analysis of covariance.

RESULTS

Figure 1 shows representative results with blood from one child before and after vaccination. Bacteria introduced into wells containing only 5% serum in tissue culture medium continued to multiply but the rates tended to decrease despite replenishment of 50% of the medium each day. In contrast bacteria introduced into wells containing leucocytes were inhibited in the first day. Substantial proportions of the bacteria were removed from leucocyte wells when they were rinsed after 24 h and during subsequent daily pulsing with lymphocytes or with medium only (sham-pulsed). The growth of the residual bacteria was variable but tended to be less after vaccination than before vaccination. Lymphocyte pulsing enhanced growth before vaccination but not afterwards.

Eight children were successfully tested before and after vaccination in this way. The bacterial growth rates were calculated and averaged across the eight individuals for each 24 h period. The averaged growth rates are shown in Table 1 together with those from the PPD positive adult laboratory volunteers tested in parallel.

The contrast during the first day between growth of the bacteria in medium only and almost complete bacteriostasis in the leucocyte wells was significant ($P < 0{\cdot}05$; paired *t*-test) both before and after vaccination. During the second day, growth in leucocyte wells was erratic so that although on average the growth rates were increased this was not significant. However, a growth inhibitory effect of vaccination was evident during the third and fourth days. This was particularly so in the lymphocyte-pulsed wells where with pre-vaccination samples the growth rates at this period had markedly increased in comparison to the rates on the first day, whereas with post-vaccination samples the growth rates had not. The growth rates at days 3 and 4 were lower after vaccination than before vaccination in seven out of the eight children. These pre-vaccination and post-vaccination results are shown graphically (Fig. 2). A comparison, by analysis of variance, of the mean pre-vaccination and post-vaccination growth rates at day 3 and day 4 showed the effect of vaccination to be highly significant ($P < 0{\cdot}01$). Surprisingly the precison of this contrast was not increased by pre-vaccination and post-vaccination samples from the same subjects. Thus, when the analysis of variance was done by the technique of taking repeated measures (on the same subjects), the value of *P* for the contrast was 0·0072 but, when it was done considering the four growth rates (before and after vaccination at 3 and 4 days) as having been measured on four different subjects, the value of *P* was 0·0073. In the sham-pulsed wells the growth rates at days 3 and 4 were also, on average, lower after vaccination than before vaccination, but the differences did not attain statistical significance.

Growth rates from the parallel tests with a total of 5 PPD-positive adults (one of whom was tested twice) followed a similar pattern, most resembling the children's post-vaccination results rather than their pre-vaccination results. Thus the growth rates tended to increase initially and then decline, although the changes were not statistically significant.

Growth rates in lymphocyte-pulsed wells were higher than in sham-pulsed wells. This was so with children, both before and after vaccination, and with PPD-positive adults. Analysis of variance showed this effect to be significant ($P < 0{\cdot}05$, taking post-vaccination and laboratory volunteer data together).

Table 1. Average growth rates in monolayers from eight children tested both before and 8 weeks after vaccination as described. Results from six tests on adult PPD-positive laboratory volunteers which were run in parallel are also shown.

		Growth rate*			
		day 1	day 2	day 3	day 4
Pre-vaccination	pulsed	−0·005 (0·076)	0·082 (0·12)	0·29† (0·037)	0·39 †(0·08)
	sham-pulsed			0·11 (0·015)	0·26 (0·063)
	cell-free	0·12 (0·035)	0·21 (0·061)	0·14 (0·061)	0·023 (0·11)
Post-vaccination	pulsed	0·080 (0·043)	0·26 (0·12)	0·15 †(0·067)	0·19 †(0·05)
	sham-pulsed			0·075 (0·064)	0·12 (0·065)
	cell-free	0·23 (0·058)	0·36 (0·108)	0·14 (0·044)	0·054 (0·042)
Laboratory controls	pulsed	0·14 (0·065)	0·23 (0·12)	0·31 (0·085)	0·077 (0·078)
	sham-pulsed			0·17 (0·084)	−0·031 (0·07)
	cell-free	0·24 (0·062)	0·34 (0·082)	0·15 (0·066)	0·072 (0·047)

*Increase in $\log_{10}$ CFU in each day. Standard error of mean in parentheses.
†$P < 0·01$ for difference between pre-vaccination and post-vaccination mean growth rates

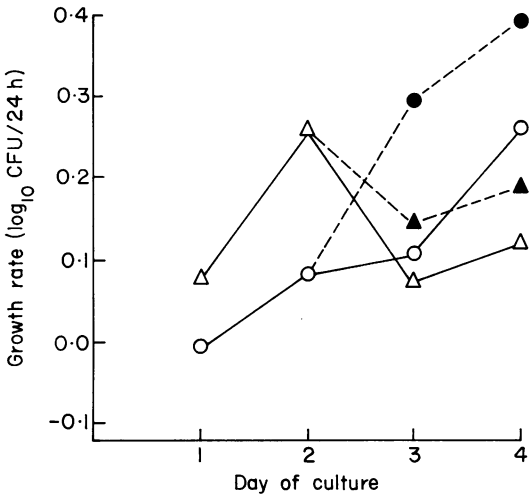

Fig. 2. Comparison between pre-vaccination (○, ●) and post-vaccination (△, ▲) growth rates showing the effect of vaccination and of lymphocyte pulsing. Data from lymphocyte-pulsed wells are represented by solid symbols with dashed lines. The data are from Table 1.

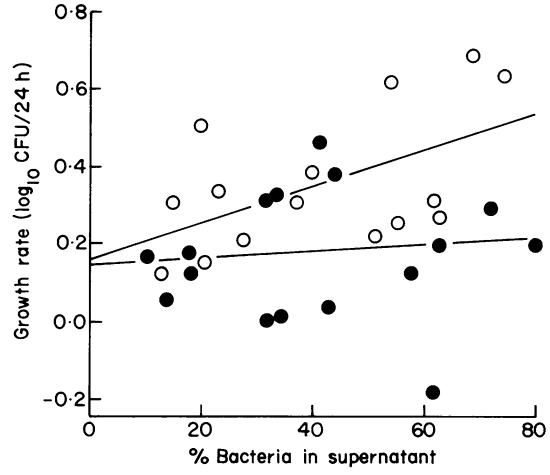

Fig. 3. Correlation between growth rate and percentage of bacteria found in monolayer supernatants. The growth rates in lymphocyte-pulsed wells during days 3 and 4 are plotted against the respective percentages of bacteria non-adherent at the end of these days. The data are from the 8 children tested before (○) or after (●) vaccination. Correlation lines were fitted by the least mean squares method.

We examined whether vaccination affected monolayer integrity, as reflected by the proportion of mycobacteria that appeared in supernatants of pulsed monolayers. This was because an effect on the ability of infected cells to adhere or to retain their bacterial load would make an apparent effect on growth rate more difficult to interpret. Figure 3 shows two things. First, that the percentage of the bacteria in the supernatants of the wells that were removed during the lymphocyte pulsing procedure at the end of days 3 and 4 (i.e. the percentage that were no longer associated with the adherent monolayer) was not affected by vaccination. Therefore vaccination reduced the growth rates without affecting the distribution of the bacteria between the adherent and non-adherent environments. Second, there was a positive correlation between growth rate and non-adherence in pre-vaccination samples. Analysis of covariance, taking pre- and post-vaccination data together, showed that the overall association between the percentage of non-adherent bacteria and growth rate was significant ($P < 0·05$) and that after correction of growth rates for differences in non-adherent proportions, the P value for the effect of vaccination on growth rates decreased from 0·007 to 0·002. In effect, this analysis, allowed for any influence non-adherence might have had on growth rates. A positive correlation between percentage non-adherence and growth rate was also evident with PPD-positive adults (data not shown). Although the percentage non-adherence tended to be smaller with adults than with children the difference was not significant.

DISCUSSION

The objective of this study was to detect an enhancement of direct anti-mycobacterial activity in peripheral blood cells after

BCG vaccination and this was achieved. The slow growth of *M. microti* during the first day after mixing with leucocytes, even from non-vaccinated children, suggests that monocytes have an innate immediate antibacterial action against *M. microti*. Since on average only about 60% of the bacteria in the wells were associated with the monolayer at the end of this period, the initial antibacterial mechanisms might include either a bacteriostatic action against extracellular bacteria or killing of a proportion of the bacteria during phagocytosis. However, the subsequent gradual increase in the growth rates of the monolayer-associated organisms in the pre-vaccination sham-pulsed wells indicates a bacteriostatic mechanism against intracellular bacteria which gradually loses effectiveness. The effect of vaccination was to prevent or retard this loss of bacteriostatic action.

We found that monocytes from children formed monolayers that were less robust, losing cells faster than those from adult laboratory volunteers (unpublished observations). When they were infected the children's monolayers correspondingly had higher proportions of bacteria in supernatants than did the monolayers from adults. However, the correlation between the appearance of a high proportion of the bacteria in monolayer supernatants and a high growth rate (Fig. 3) suggests that monocytes that lost bacteriostatic activity also lost the property of surface adherence. It was important therefore that with children's monolayers we found that the bacteria were not more likely to occur in monolayer supernatants before vaccination than after. This meant that the bacteriostasis-promoting effect of vaccination was not due to vaccination having enhanced the in-vitro survival of macrophages which individually had an unaltered bacteriostatic activity. On the contrary, vaccination enhanced bacteriostasis without affecting monolayer integrity.

The increase in stasis after vaccination may have been mediated, at least in part, by the initial exposure of the monocytes to sensitized lymphocytes during the first day in culture. The cells were not fractionated and any sensitized lymphocytes in infected wells would presumably be stimulated by *M. microti* antigen. However the subsequent pulses of exposure to cells that had been incubated with *M. tuberculosis* antigen had the unexpected effect of accelerating mycobacterial growth rates (pulsed compared to sham-pulsed at days 3 and 4). Since this was pronounced with pre-vaccination blood samples it was apparently not dependent upon sensitization by vaccination. Thus there might be two opposing effects of lymphocyte exposure: one operating only after vaccination to bring about an enhancement of bacteriostasis, the other causing impairment of bacteriostasis and operating both before and after vaccination. When the actual cells and cell products responsible for these two opposing phenomena are identified it will be interesting to see if different lymphocyte subsets responding to different mycobacterial components are responsible for each.

There is evidence that bacteriostasis in macrophages may be a major component of immunity against tuberculosis in the mouse (Hart & Rees, 1960; Rook *et al.*, 1986; Flesch & Kaufmann, 1987) but the situation is much less clear in man. Our observation of initial bacteriostasis in fresh monocytes differs from that of Douvas *et al.* (1986) where human monocytes were found to be permissive to growth of *M. tuberculosis* when they were first isolated. In that study growth during the first 24 h of infection was not assessed but the cells became bacteriostatic if they were cultured for 3 days before they were infected. Our finding that vaccination reinforced bacteriostasis rather than inducing killing might follow from the way phagocytosis would have largely preceded activation by lymphocytes in our test. This does not preclude finding killing when, conversely, activation precedes phagocytosis. Indeed, Khor *et al.* (1986) found that gamma-interferon can activate murine macrophages for both bactericidal and bacteriostatic action against *M. microti*, the bactericidal effect being seen only when the cells were activated before phagocytosis.

Whether the enhanced anti-mycobacterial action detected here after vaccination is an adequate guide to the acquisition of protective immunity against tuberculosis in man remains to be established. This can only be done by repeating the test in different populations in whom different effects of BCG vaccination on actual incidence of disease can be measured. If it is a guide, then a simplified form of the test may be a means of quickly identifying human populations in whom BCG vaccination will or will not be effective and could aid in assessing the value of new replacement vaccines.

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