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Protective response in guineapigs exposed to *Mycobacterium avium intracellulare/ M. scrofulaceum,* BCG & south Indian isolates of *M. tuberculosis*

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The protective immunity resulting from exposure to nontuberculous mycobacteria (NTM), BCG and virulent mycobacteria in different sequences was studied in the guineapig model employing strains prevalent in the south Indian BCG trial area and time kinetics to observe the immuno-modulation. The findings suggest that during the early course of challenge infection in guineapigs there was no interference with the immunity due to BCG, by prior exposure to NTM. In the animals sensitised with *M. avium intracellulare* before immunisation, the challenge infection was localised and confined to the site of inoculation, and only a few organisms reached the spleen.. However, at the later stages of the infection, as seen by the spleen viable counts at 12 wk, it appeared that the barrier at the localised site of infection may not be intact in the animals with prior exposure to NTM, and a few organisms disseminate to the spleen.

Key words BCG-immunomodulation-non-tuberculous mycobacteria-spleen viable counts-south Indian isolates

Interactions with the immune responses to other mycobacterial infections still remains one of the most popular explanations for BCG's varying efficacy[1,2]. Palmer and associates[3,4] showed in animal experiments, and in studies of US Navy personnel, that infections with certain non-tuberculous mycobacteria (NTM) could impart some protection against infection with the tubercle bacillus and such naturally acquired protection could mask the protection due to BCG vaccination.

In 1970, Abrahams[5] pointed out that the first mycobacterial infection may set the antigenic reaction pattern and in the 1980s Stanford, Rook and associates[6-8] proposed that exposure to NTM could result in two types of cell-mediated responses, the 'Listeria type' and the 'Koch type'. Which of these two types of responses is evoked depended, among other factors, on the mycobacterial species inducing the response and the immunomodulating cells and the pathway brought into play. They further proposed that the 'Listeria type' of response enhances the protective effect of subsequent vaccination with BCG while the 'Koch type' response opposes the protective effect of BCG. BCG vaccination of a person with a pre-existing Koch-like response will temporarily boost this response, but completely fail to reconvert to Listeria-like responsiveness or induce protection from pathogenic challenge. According to them, this is likely to have been the situation in the south Indian trial[9,10]. However, attempts by others to demonstrate that prior infection with any of the mycobacteria induced a suppressive effect against BCG have failed[11-14].

The findings of Raj Narain *et al*[15] that there is widespread sensitisation to PPD-B in the BCG trial area of south India, and the findings of Paramasivan *et al*[16] that *Mycobacterium avium-intracellulare-scrofulaceum* (MAIS) strains are among the most frequently isolated from the sputum of patients from this area suggest the possibility that MAIS strains may be widely prevalent in this region. Also, nearly 70 per cent of *M. tuberculosis* isolates from this area are of low virulence for guineapigs and have been designated as the south Indian variant (SIV)[17-20]. Exposure of the population in this area to these NTM, BCG and *M. tuberculosis* is very likely to be occurring in different possible sequences. The present study was undertaken in the guineapig model employing strains prevalent in the region and to examine the time kinetics with reference to the protective immunity resulting from such an exposure.

Material & Methods

Animals : Twenty four random-bred guineapigs of the M-strain[21] from the animal house of the Tuberculosis Research Centre (TRC), Madras were used in this experiment.

The guineapigs were divided into five groups, groups A to E, with 6 animals in groups C to E, and 3 each in groups A and B since they did not have further subgroups.

Strains of mycobacteria : The strains of mycobacteria used in the experiment were, *M. avium intracellulare* (standard TMC strain 1403 maintained at TRC), *M. scrofulaceum* strain isolated from a patient and maintained at TRC), BCG (standard strain Glaxo maintained at TRC) and *M. tuberculosis* south Indian variant (strain isolated from a south Indian patient before treatment and maintained at TRC).

Preparation of the inoculum : Growth from Lowenstein Jensen (LJ) slope was inoculated into Middlebrook's 7H9 liquid medium (Difco) containing albumin and dextrose. The 7H9 cultures were incubated for 2 wk at 37°C, centrifuged at 3000 rpm (Beckman J-6B) for 20 min at room temperature and the pellet was resuspended in sterile saline (0.85%) containing 0.05 per cent Tween 80. The total number of organisms per ml in this suspension was counted under the microscope using a Thoma bacterial counting chamber (Gallenkamp, London) and was adjusted to 5 x 10^6/ml using Tween saline to prepare the standard suspensions for inoculation. After preparation, the suspensions were distributed into glass universals in 5 ml aliquots and stored at -20°C until use.

Sensitisation, immunisation and challenge : For sensitisation, immunisation and challenge, inocula containing a total number of 1 x 10^6 (total count) of each organism in 0.2 ml saline with Tween (0.05%) were given subcutaneously to the guineapigs on the inner side of the thigh. Different sensitisation, immunisation and challenge schedules were followed for each group of guineapigs as shown in Table I. The guineapigs in group A were not subjected to prior sensitisation or immunisation but were directly injected with *M. tuberculosis* south Indian variant (SIV). The guineapigs in group B were first immunised with BCG. After six wk, they were injected with SIV. The guineapigs in group C were also first immunised with BCG. After six wk, half of these animals were given *M. avium intracellulare* (Mai) and the others were given *M. scrofulaceum.* After another six wk, they were injected with SIV. Half of the guineapigs in group D were sensitised with Mai,

Table I. Experimental design for viable count in spleen at 12 wk after challenge

Week	Group A	Group B	Group C	Group D	Group E
0	SIV	BCG	BCG	NTM	NTM
	1	1	1	1	1
6	None	SIV	NTM	SIV	BCG
	1	1	1	I	1
12	VC	1	SIV	1	SIV
		1	1	1	1
18		VC	1	VC	1
			1		I
24			VC		VC

SIV, *M. tuberculosis* south Indian strain

NTM, nontuberculous mycobacteria *(M. avium intracellulare/ M. scrofulaceum)*

VC, viable count in spleen

and the other half with *M. scrofulaceum* first. After six wk, they were injected with SIV. The animals in group E were also sensitised with Mai or *M. scrofulaceum* first. After six weeks they were given BCG and after another six wk they were infected with SIV.

Sacrifice and spleen viable count : The guineapigs were sacrificed using chloroform vapour 12 wk after challenge. Autopsy was done. A portion of the spleen was collected and homogenised in 5 ml sterile distilled water in a motor driven teflon/glass grinder. Viable count was set up on LJ using 10 µl of this neat suspension and 5 serial ten-fold dilutions, and colony forming units (cfu) per spleen were calculated. From this, the average cfu/spleen for each subgroup was calculated. The organisms recovered from the spleen in the viable count culture were confirmed to be *M. tuberculosis* by niacin test.

Time kinetics experiments : To examine the kinetics of the early course of challenge infection this experiment was undertaken.

Guineapigs : Twenty four random-bred M-strain guineapigs from the animal house of TRC were used in this experiment. These were divided into three groups with 8 animals in each group.

Preparation of the inoculum : The suspension for inoculation was prepared fresh every time. Growth from 2 wk old cultures of the strains on LJ was transferred with a wire loop to pre-weighed Bijou bottles containing 0.2 ml sterile distilled water and glass beads. The bottles were weighed again to calculate the weight of the organisms, shaken for 1 min in a mechanical shaker and the resulting suspension was diluted using sterile distilled water to contain 1 mg of the organism in the required volume.

Sensitisation, immunisation and challenge schedule: For sensitisation and immunisation, 1 mg moist weight of the organism in 0.1 ml sterile distilled water was injected intradermally in the inner side of the thigh. For challenging, 1 mg moist weight of the challenge organism in 0.5 ml sterile distilled water was injected intramuscularly into the thigh. The sensitisation, immunisation and challenge schedule followed for each group of guineapigs is shown in Table II. The guineapigs in group 1 served as controls and were challenged with *M. tuberculosis* south Indian low virulent strain (SIV) without prior sensitisation or immunisation. The guineapigs in group 2 were immunised first with BCG. After 6 wk, these animals were challenged with SIV. The guineapigs in group 3 were sensitised first by injecting with Mai. After 6 wk, these animals were given BCG, and 6 wk later were challenged with SIV.

Skin tests : All the guineapigs used in the time kinetics experiment were skin tested with 5 µg of PPD-RT22 in 0.1 ml normal saline one wk before they were challenged. The skin test was administered on the dorsal side of the animal after shaving the selected area. The skin test readings were taken at 48 h.

Sacrifice and spleen viable counts : At 2, 4, 6 and 8 wk after challenge, two animals from each subgroup were sacrificed. Spleen viable counts were set up as before.

Statistical analysis : Student's 't' test was used to test the differences in the mean cfu in the spleen between groups,. and to test the significance of correlation coefficient between skin test reactivity and cfu in spleen.

Results

The viable count in the spleen 12 wk after challenge are given in Table III. In the control animals challenged with SIV without prior sensitisation or immunisation, 5.56 log colony forming units (cfu) could be recovered from the spleen 12 wk after challenge. While none of the animals which had been immunised with BCG

Table II. Experimental design for kinetics of challenge infection

Week	Group 1	Group 2	Group 3
0			Mai
6		BCG	BCG
12	SIV	SIV	SIV
14-20	VC	VC	VC

SIV, *M. tuberculosis* south Indian strain

VC, viable count in spleen, these were studied at 2, 4, 6, 8 wk after challenge

Mai, *M. avium intracellulare*

Table III. Viable counts in the spleen of guineapigs 12 wk after challenge with *M. tuberculosis* south Indian strain

Guinea-pig no.	Group	cfu in spleen	Mean cfu (log) in spleen/ group
1	SIV	1077000	
2	SIV	8620	5.56
3	SIV	0	
4	BCG-SIV	0	
5	BCG-SIV	0	0.00*
6	BCG-SIV	0	
7	Mai-SIV	0	
8	Mai-SIV	431	2.16
9	Mai-SIV	0	
10	Msc-SIV	0	
11	Msc-SIV	646	2.33
12	Msc-SIV	0	
13	BCG-Mai-SIV	0	
14	BCG-Mai-SIV	0	0.00*
15	BCG-Mai-SIV	0	
16	BCG-Msc-SIV	0	
17	BCG-Msc-SIV	0	0.00*
18	BCG-Msc-SIV	0	
19	Mai-BCG-SIV	0	
20	Mai-BCG-SIV	0	2.16
21	Mai-BCG-SIV	431	
22	Msc-BCG-SIV	0	
23	Msc-BCG-SIV	215	2.33
24	Msc-BCG-SIV	431	

SIV, *M. tuberculosis* south Indian strain
Mai, *M. avium intracellulare*
Msc, *M. scrofulaceum*
cfu, colony forming units
* 0.00 actually means that there was no growth in culture and implies that the log cfu in spleen was less than 1.85 which was the minimum detectable level in these experiments

before challenge (BCG-SIV) had detectable number of organisms in the spleen 12 wk after challenge, those which had been given Mai, or *M. scrofulaceum* before challenge (Mai-SIV and Msc-SIV) had log cfu of 2.16 and 2.33, respectively. These numbers, however, were very much lower than the number of organisms (5.56 log cfu) recovered from the spleens of control animals challenged directly with a similar dose of SJV.

Among the animals challenged after they had been given BCG followed by NTM, or NTM followed by BCG, none of the animals which were exposed to BCG first had detectable number of organisms in the spleen 12 wk after challenge. On the other hand, among the animals exposed to NTM first, the animals exposed to Mai first (Mai-BCG-SIV) had log cfu of 2.16, and animals exposed to *M. scrofulaceum* first (Msc-BCG-SIV) had log cfu of 2.33 in the spleen 12 wk after challenge.

However, none of the differences between the different groups of guineapigs in the number of viable organisms in the spleen at 12 wk after challenge, were statistically significant ($P < 0.05$).

Time kinetics experiments: Skin test : The results of the skin test reactivity to PPD-RT22 are given in Table IV. The skin test reactivity before challenge was minimal (1.94 mm) in animals which had not been exposed to any mycobacteria. In the animals exposed to BCG alone, and in the animals exposed to Mai and BCG, the skin test response was high (8.69 mm and 10.5 mm, respectively).

Viable count in spleen : In the animals directly challenged with SIV, the maximum number of organisms (4.23 log cfu) was seen in the spleen at 2 wk after challenge. The number of organisms in the spleen was lower at 4 wk (2.44 log cfu) and became undetectable by 6 wk (Table IV). In the animals challenged with SIV after they had been immunised with BCG (BCG-SIV), or sensitised with Mai and then immunised with BCG (Mai-BCG-SIV) also, the maximum viable counts were seen at 2 wk after challenge (3.10 and 2.99 log cfu, respectively). These numbers, however, were significantly less ($P < 0.05$) than the maximum log viable counts in the directly challenged animals. The challenge organisms could not be detected in the spleen 6 wk after, challenge in animals immunised with BCG (BCG-SIV) and by the 4th wk itself in animals which were sensitised with Mai before immunisation with BCG (Mai-BCG-SIV).

A significant negative correlation was observed between skin test reactivity to PPD-RT22 at 48 h and viable count in spleen at 2 wk after challenge ($r = 0.94$; $P < 0.05$).

Discussion

The findings of the present study indicate that

Table IV. Time kinetics - viable count in spleen of guineapigs challenged with *M. tuberculosis* south Indian strain

Guinea-pig no.	Group	Skin test reading (mm)	Viable count in spleen		
			Time after challenge (wk)	cfu in spleen	Mean cfu (log) in spleen
1	SIV	2	2	25400	4.23
2	SIV	0	2	8490	
3	SIV	2	4	0	2.44
4	SIV	0	4	550	
5	SIV	2.5	6	0	0.00*
6	SIV	3	6	0	
7	SIV	2	8	0	0.00*
8	SIV	4	8	0	
	Mean skin test reactivity (mm)	1.94			
9	BCG-SIV	9	2	0	3.10
10	BCG-SIV	12.5	2	2520	
11	BCG-SIV	12	4	0	2.61
12	BCG-SIV	12	4	816	
13	BCG-SIV	7	6	0	0.00*
14	BCG-SIV	8	6	0	
15	BCG-SIV	4	8	0	0.00*
16	BCG-SIV	5	8	0	
	Mean skin test reactivity (mm)	8.69			
17	Mai-BCG-SIV	7	2	1945	2.99
18	Mai-BCG-SIV	13	2	0	
19	Mai-BCG-SIV	9	4	0	0.00*
20	Mai-BCG-SIV	12	4	0	
21	Mai-BCG-SIV	10	6	0	0.00*
22	Mai-BCG-SIV	10	6	0	
23	Mai-BCG-SIV	11	8	0	0.00*
24	Mai-BCG-SIV	12	8	0	
	Mean skin test reactivity (mm)	10.5			

SIV, *M. tuberculosis* south Indian strain
Mai, *M. avium intracellulare*
cfu, colony forming units
* 0.00 actually means that there was no growth in culture and implies that the log cfu in spleen was less than 1.85 which was the minimum detectable level in these experiments

in guineapigs there is no interference to the immunity due to BCG by prior exposure to NTM in the early coupe of challenge infection. In these animals, as in BCG vaccinated animals, the challenge infection is well contained during the early course of infection and very few organisms reach the spleen from where they are eliminated in a few wk. However, at the later stages of the infection, as seen by the spleen viable counts at 12 wk, it appears that the barrier at the localised site of infection may not be intact in the animals with prior exposure to NTM, and a few organisms disseminate to the spleen. What happens after this time point cannot be ascertained from the present study, but need studies with longer experimental duration for an answer. It is also possible that multiple exposures to NTM could prevent the waning off of immune response resulting from a single exposure. The dissemination taking place during the later course of infection may probably be prevented by such multiple exposures. Multiple small dose exposure to tubercle bacilli, as happens

in nature, may also lead to different results. Further studies are needed to examine these possibilities.

Based on evidence which indicates that BCG vaccination protects humans by interfering with the haematogenous spread of bacilli, Wiegeshaus and Smith[22] have proposed that a rational animal model is one in which interference with haematogenous spread of bacilli is measured in animals infected by the respiratory route with small numbers of bacilli as occurring in human beings. The animal model used in the present study differs from the rational model in that respiratory route has not been used. Further, the effect of multiple small dose exposure has not been studied. However, because of its design this model also measures only the changes in the haematogenous spread of bacilli and thus protection.

Various mechanisms could be responsible for the modulation seen during the later course of infection in the animals exposed to NTM first followed by BCG vaccination and then challenged. At the initial stages, these animals are able to contain the challenge infection to the site of inoculation. However, at these sites the challenge organisms might multiply later and reach high local concentrations. The delayed type hypersensitivity (DTH) could then become harmful and cause tissue destruction and release of bacilli[23].

It is also possible that the small numbers of organisms detected in the spleen at 12 wk after challenge could be dormant bacilli released from the local site from time to time. Lowrie and Andrew[24] suggest that it has become increasingly possible that immunological activation of macrophages, apart from resulting in killing, can also result in reduced intracellular mycobacterial multiplication leading to stasis. In such a situation, the beneficial effect of cytotoxic lymphocytes in resistance, could be through the release of bacilli from cells in which they are dormant so that they become exposed to the killing mechanisms[24].

As proposed by Collins[25], suppressor cells may be responsible for the inability of the host defences to recognise the continuing presence of sensitising antigens of the organism and thus contribute to the persistence of infection within the apparently normal immunocompetent host. As proposed by Tsumyguchi *et al*[26], infection with NTM could generate suppressor cells which act by inhibiting interleukin 2 (IL-2) production or, as Ellner and Wallis[27] propose, exaggerated production of IL-1 may be part of the mechanism of suppression.

The results of the present study thus suggest that certain modulation of the protective immunity due to BCG was probably taking place in animals exposed to NTM first. However, these results have to be confirmed in a larger scale study. In populations of endemic areas of tuberculosis with high prevalence of NTM, prior exposure to NTM may have similar modulation over the immunity due to BCG in the later course of infection. This may explain, at least partly, the varying efficacy of BCG seen in the different vaccination trials.

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