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IgG ANTIBODIES AGAINST ANTIGENS OF VARIOUS MYCOBACTERIAL SPECIES IN CHILDREN AND IN PRE- AND POST-BCG YOUNG ADULTS

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Summary: **IgG antibodies against antigens of various mycobacteria were estimated by ELISA in serum samples collected (a) from 36 children (mean age 4.4 years) belonging to Koppur village in the south Indian BCG Trial area, (b) before and after BCG vaccination of 13 young individuals (mean age 16.5 years) belonging to Trivellore in the same area and (c) before and after BCG vaccination from 20 young British subjects (mean age 14.5 years). In the Koppur children, the antibody levels were highest against *M. scrofulaceum* and *M. avium* and lowest against *M. bovis* and *M. tuberculosis* H37Rv. In these children, there was no correlation between antibody levels and tuberculin reactivity. In the Trivellore subjects, antibody levels were highest against *M. bovis BCG* and *M. gordonae,* and lowest against PPD RT22 and *M. terrae* and none of the differences in the antibody levels against individual antigens between the pre- and post-BCG serum samples was statistically Significant (p >.05). The British subjects had the highest levels against *M. tuberculosis* 7219 while the lowest levels were against *M. kansasii* and *M. tuberculosis* 51; after BCG vaccination the antibody levels were selectively increased against *M. tuberculosis* 7219, *M. flavescens* and *M. gordornae* (p <0.05).**

Introduction

The protective effect of BCG in controlled trials has ranged from none in a south Indian trial to almost 80 per cent in a British trial[1]. It is likely that the low protective effect in south India compared to Britain is at least partly due to greater prior sensitisation to environmental mycobacreria in south India which may obviate or block the effect of subsequent vaccination.[2-4] The degree of sensitization to mycobacterial antigens varies from country to country probably because of the relative prevalences of non-tuberculous mycobacteria (NTM) in the environment and genetic factors, or even within the same area due to differences in age and social behaviour patterns[5]. Presumably, in south India there is a widespread infection with NTM in children from very early age, and it has been reported that among the NTM, *M.avium intracellulare, M. terrae* and *M.scrofulaceum* are the species most frequently isolated from the sputum of subjects belonging to this area[6].

Although it is generally accepted that humoral immune response resulting from this sensitisation does not provide adequate protection against tuberculosis, the antimycobacterial antibodies may be of importance in modulating the host's delayed type hypersensitivity and protective immune response through various mechanisms[7-9] and in the development of diagnostic methods based on antibody detection. We have measured the IgG

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antibody levels to various mycobacterial species by ELISA in serum samples from a group of children and young individuals belonging to the south Indian BCG trial area and from a group of young British subjects to obtain information on the sensitisation patterns to mycobacterial antigens in these three groups to find out if there is any association between antibody levels and tuberculin reactivity status in children belonging to this area, and to compare the changes, if any, and the differences in changes in the antibody levels after BCG vaccination in young individuals belonging to this area and Britain.

Material and Methods

Serum samples : Serum samples collected from 36 children from Koppur village in the south Indian BCG trial area were used. The age of these children ranged from 2 to 6 years and the mean was 4.4 years. The tuberculin reactivity to PPD-S ranged from 0 to 30mm and the mean was 7.5mm. The children could be divided into three different tuberculin reactivity groups; 18 had 0-3mm reactivity, 8 had 4-10mm reactivity and 10 had >10mm reactivity, representing low, intermediate and high level of reactivity to PPD-S.

Serum samples, collected before as well as 8 weeks after BCG vaccination and tuberculin conversion, from 13 young individuals from Trivellore in the same geographic area (mean age 16.5 yrs) and 20 young British subjects (mean age, 14.5 yrs) were also included in the present study. The pre-BCG Mantoux reaction to PPD-S was <8mm to 3TU in the young Indians and <4mm to 4TU in the young British subjects. The post-BCG/tuberculin conversion reaction was >12 mm to 1 TU in these individuals.

Serum samples from the young British subjects were provided by D.B. Lowrie, National Institute of Medical Research, Mill Hill, London.

Antigens : The IgG antibody levels in the serum samples were estimated by ELISA against PPD-RT 22 and mycobacterial sonicate supernatant antigens listed in Tables 1 and 2.

The sonicate antigens were prepared by C.N. Paramasivan in the Royal Postgraduate Medical School, London, as described earlier[10]. PPD-RT 22 was obtained from the BCG Laboratory, Madras.

ELISA procedure : ELISA to estimate the antimycobacterial antibodies of class IgG in the serum samples was done as described by Narayanan et al[11]. Briefly, 'antigen coating of microwell plates (Gibco/Nunc Cat. No. 439454) was carried out using 0.1ml of 5mcg/ml solution of the antigen in carbonate buffer (0.06M, pH 9.6) per well. Each serum sample was divided into two aliquots and given different code numbers. Each coded serum sample was tested at 1/40 and 1/80 dilutions on 15 plates, each plate coated with a different mycobacterial antigen. A 1/100 dilution of anti-human IgG peroxidase conjugale (Sigma Cat. No. A-6029) was used as the secondary antibody and orthophenylene diamine (OPD) was used as the substrate. The optical density (OD) of the resultant reaction in each well was read at 490nm in a Biotek ELISA plate reader.

Statistical analysis: From the OD values from duplicate samples tested, average values were calculated for each dilution of the serum. The mean antibody levels against individual antigens for the different tuberculin reactivity groups among the children were compared by unpaired t-test. Mean antibody levels against individual antigens for the pre- and post-vaccination samples were compared by paired t-test. A one-way ANOVA was carried out for all the antigens in the three tuberculin reactivity groups, and the means were tested for increasing trend after adjusting for the antigens as covariant.

Results

The mean IgG antibody levels in OD units at 1/40 dilutions of serum samples from the 36 Koppur children belonging to the three different tuberculin reactivity groups are presented in Table 1. Antibody levels were highest against *M.scrofulaceum* and *M.avium* serotype 8 while the lowest levels were against *M.tuberculosis* H37Rv in all the three groups. The ranking of antibody levels (from highest to lowest) was also similar in these three groups: it was *M.scrofulaceum* followed by *M.avium intracelluare* serotype 8,

Table 1. *Mean antibody levels and standard deviations in OD units at 1/40 dilution*

Serum samples from children (mean age 4.4 years) belonging to different tuberculin reactivity groups

Tuberculin reactivity Group mean din.		Antigen Mtb H37Rv	Mtb 51	Mtb 7219	Mbo	BCG	Mkan	Mscr	Mai 8	Mai 16	Mter	Mfla	Mgor	Mfor	Mche	PPD
0-3mm 0.33 (n=18)	Mean	.128	.284	.222	.175	.216	.199	.488	.386	.218	.234	.200	.218	.230	.232	.234
	SD	.050	.127	.096	.069	.091	.070	.133	.069	.045	.068	.036	.040	.042	.042	.044
p<.001																
4-10mm 8.62 (n=8)	Mean	.138	.265	.220	.172	.194	.194	.425	.370	.217	.225	.184	201	.218	.222	.222
	SD	.059	.159	.110	.076	.079	.088	.153	.091	.038	.078	.026	.027	.033	.038	.035
p<.001																
>10mm 19.5 (n=10)	Mean	.150	.300	.251	.186	.227	.201	.476	.358	.215	.259	.189	.202	.204	.223	.218
	SD	.068	.172	.105	.095	.106	.096	.135	.084	.061	.050	.038	.044	.027	.038	.024
All Children (n=36)	Mean	.136	.284	.229	.177	.214	.198	.471	.375	.217	.239	.193	.210	.220	.227	.227
	SD	.056	.144	.100	.076	.091	.079	.137	.077	.047	.065	.035	.039	.037	.040	.038

Mtb H37Rv = *M.tuberculosis* H37Rv (laboratory strain), Mtb 51 = *M.tuberculosis* 51 (British strain), Mtb 7219 = *M.tuberculosis* 7219 (South Indian strain), Mbo = *M.bovis* (NCTC 5693), BCG = *M.bovis* BCG (Glaxo), Mkan = *M. Kansasii* (NCTC 10268) Mscr = *M. Scrofulaceum* (NCTC 10803) Mai8 = *M. avium intracellulare* serotype 8 (NCTC 10610), Mai16 = *M.avium intracellulare* serotype 16 (NCTC 10425), Mter = *M. terrae* (NCTC 10856), Mfla = *M.flavescens* (NCTC 10271), Mgor = *M.gordonae* (NCTC 10267). Mfor = *M.fortuitum* (NCTC 10394), Mche = *M.chelonei* (NCTC 10882), PPD = PPD RT22.

Table 2. *Mean antibody levels and standard deviations in OD units at 1/40 dilution*

Pre- and post-BCG serum samples from young Indian and British subjects

BCG status		Antigen*														
		Mtb H37Rv	Mtb 51	Mtb 7219	Mbo	BCG	Mkan	Mscr	Mai 8	Mai 16	Mter	Mfla	Mgor	Mfor	Mche	PPD
Young Indian Subjects (n=13)																
Pre-BCG	Mean	.320	.378	.346	.364	.468	.342	.348	.368	.306	.245	.273	.456	.408	.396	.260
	SD	.090	.109	.136	.139	.065	.076	.088	.052	.108	.058	.067	.094	.094	.102	.082
POST BCG	Mean	.321	.379	.340	.374	.469	.323	.361	.375	.301	.236	.262	.454	.405	.401	.265
	SD	.029	.034	.120	.125	.055	.052	.085	.053	.074	.055	.044	.087	.079	.095	.074
Young British Subjects (n=20)																
PRE-BCG	Mean	.334	.262	.483	.302	.432	.283	.296	.374	.434	.406	.358	.437	.360	.447	.442
	SD	.090	.075	.112	.065	.083	.083	.076	.053	.077	.079	.060	.070	.071	.100	.095
				p<.05								p<.01	p<.01			
POST-BCG	Mean	.343	.267	.510	.397	.429	.290	.315	.375	.449	.417	.394	.465	.364	.429	.406
	SD	.087	.075	.107	.052	.084	.068	.076	.052	.082	.076	.080	.073	.061	.095	.076

* For the full names of antigens see footnote under Table 1

M. tuberculosis 51, *M.terrae.* PPD-RT 22, *M.chelonei, M.fortuitum, M.tuberculosis* 7219, *M.avium intracellulare* serotype 16, *M.gordonae,* BCG, *M.flavescens. M.kansasii, M.bovis* and *M.tuberculosis* H37Rv in the 0-3mm group; the same pattern with only the positions of *M.tuberculosis* 7219 and *M.fortuitum,* and *M.kansasii* and *M.flavescens* interchanged in the 4-10mm group; and, again, a similar pattern with *M.tuberculosis* 7219 and BCG moving up in the order and the positions of *M.chelonei.* PPD-RT 22, *M.fortuitum* and *M.gordonae* changed in the >10mm group. Similar results were obtained with 1/80 dilutions of the serum samples (data not presented). The mean tuberculin reactivity in the three groups of children were 0.33, 8.62 and 19.5mm. Unpaired t-test showed that there were no significant differences in the mean antibody levels against individual antigens between the three tuberculin reactivity groups ($p>0.05$). In one-way ANOVA, no significant variation was seen, and there was no statistically significant increasing trend.

Of the Koppur children, 23 were male and 12 were female. The mean tuberculin reactivities in the male and female children were 6.70 and 9.08mm. respectively. The difference in the tuberculin reactivities of the two groups was not Statistically significant ($p>0.05$). The differences between the males and females in the mean antibody levels against individual mycobacterial antigens were also not statistically significant ($p>0.05$).

The pattern of antibody levels at 1/40 dilution of serum samples from the young individuals from Trivellore were different from those in the Koppur children (Table 2). In the pre-BCG young individuals from Trivellore, the antibody levels were highest against *M. bovis* BCG and *M.gordonae.* The lowest levels at this dilution were seen against PPD-RT22 and *M.terrae.* At 1/80 dilution also, similar results were obtained (data not presented). In the young Indian subjects, the mean antibody levels against individual antigens in post-BCG; serum samples were similar to the levels in pre-BCG samples; the order of antibody levels to the 15 antigens was also the same in post-BCG samples except that *M.flavescens* and PPD-RT22 had interchanged their positions. Results with 1/80 dilutions (data not shown) were very little different from those with 1/40 dilutions. Paired t-test revealed that none of the differences in the mean antibody levels against individual antigens between the pre- and post-BCG serum samples at 1/40 or 1/80 dilution was statistically significant ($p>0.05$).

The pattern of antibody levels was different in the young British subjects. The highest levels of antibodies at 1/40 dilution of both pre- and post-BCG/tuberculin conversion serum samples were found against *M.tuberculosis* 7219. The lowest levels were seen against *M.kansasii* and *M.tuberculosis* 51. Essentially, identical patterns were obtained with 1/80 dilutions of the serum samples also (data not shown).

In the young British subjects, the post-BCG serum samples had significantly higher mean antibody levels than pre-BCG samples against *M.tuberculosis* 7219, *M.flavescens* and *M.gordonae* at 1/40 dilution (Table 2) and to *M.gordonae* (.282 and .313 in the pre-and post-BCG samples. respectively) at 1/80 dilution also ($p<0.05$). The mean antibody levels against the other antigens. individually, were similar (differences not statistically significant, $p>0.05$) in the pre-and post-BCG samples.

Discussion

Even though the sample size is perhaps too small to permit generalization, several interesting observations are evident from this study. It has been reported earlier that *M.avium intracellulare* and *M.scrofulaceum* are among the NTM species most frequently isolated from the sputum of subjects belonging to the south Indian BCG trial area[6]. The prominence of antibody response to *M.avium intracellulare* and *M.scrofulaceum* in subjects from this area in the present study also suggests that these NTM species are most likely to be the ones responsible for the early appearance and wide prevalence of DTH to PPD-B in this area.

The pattern of antimycobacterial antibodies in the young individuals (mean age 16.5 yrs) from Trivellore was different from that in the Koppur children (mean age 3.4 years). Age, along with

any differences in the profile of environmental mycobacteria, could probably account for the difference in the patterns of antibody levels between the two groups of subjects.

An interesting observation in the present study was the relatively low levels of antibodies seen against the antigens to which the older subjects were likely to have had more exposure as in the case of *M.tuberculosis* 7219 (prepared from a south Indian low virulence strain) and *M.tuberculosis* 51 (prepared from a British strain) in the Indian and British subjects, respectively.

The present study showed that in the Koppur children there were no significant differences in the mean antibody levels against individual antigens including PPD-RT 22 between the three tuberculin reactivity groups (0-3,3-10 and >10mm) indicating that tuberculin reactivity may be independent of the antimycobacterial antibody levels in these children. Many earlier studies by others have examined the relationship between tuberculin reactivity status and antimycobacterial antibody levels with similar findings[12-15]. Correlation between tuberculin reactivity and antibody levels have also been reported by others[16-17].

The relationship between BCG vaccination and antibody levels has been examined by many workers. Raheman et al[13] in India did not find any correlation. Some of the studies conducted abroad have also reported similar findings[15,17,20]. On the other hand, positive correlation between BCG vaccination status and antibody levels have been reported by others[21-24]. Based on such evidence from literature, it has been concluded by Grange[7] that BCG vaccination appeared to induce only a transient antibody response.

The differences seen in the present study between the Indian and British subjects in the humoral immune response to BCG vaccination could be due to the differences in the pre-existing sensitisation patterns between these two groups[4,5]. Prior contact with shared mycobacterial antigens could accelerate subsequent responses to these antigens and may also suppress the formation of antibodies to newly introduced specific antigens by the phenomenon of antigenic competition[7]. Genetic differences between the two groups studied could also be involved acting through the HLA class 2 immune response genes[25].

The differences in the patterns of antibodies observed in the present study between the Indian and British subjects could be due to differences in the degree of exposure and in the mycobacterial antigens encountered by these two groups[5]. According to Grange[7], differences in antibody levels between individuals could be due to differences in prior exposure of antigens different antigens being processed differently by macrophages, the amount of antigens, local tissue reactions to the antigens, formation of immune complex, general state of nutrition, interaction of other immune responses, presence of immunosuppressive factors and genetic factors. The relevance of the differences seen between the British subjects and Indian subjects from the BCG trial area in the humoral immune response to BCG vaccination and pattern of antibodies is not clear. Stanford et al[26] examined the value of multiple skin testing. lymphocyte transformation test and ELISA of antibodies to mycobacterial antigens as correlates of protection. Of the three types of tests assessed, only skin testing appeared to be of any value as a measure of protection. The question whether the differences in the antibody patterns and response to BCG vaccination in the Indian and British subjects seen in the present study have any such prognostic significance needs to be addressed further.

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