

Childhood atopy and allergic disease and skin test responses to environmental mycobacteria in rural Crete: a cross-sectional survey

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Summary

Background Through its powerful immunoregulatory effects, infection with atypical mycobacteria may exert a protective effect on the development of childhood allergic disease.

Objective To examine the relationship between childhood atopy or allergic disease and previous infection with four species of atypical mycobacteria.

Methods Eight hundred and six children aged 8–18 years and living in rural Crete – most of whom had had previous BCG immunization – underwent skin prick testing with 10 aeroallergens; their parents completed a standardized questionnaire relating to allergic disease. No less than 8 weeks later each child underwent intradermal skin tests with 0.1 mL solutions of four selected mycobacterial reagents (Aviumin C, Gordonin, Chelonin and Ranin I).

Results Twenty-three percent of children were atopic on skin prick testing; far fewer had symptoms of asthma (5%) or hayfever in conjunction with a positive prick test to pollens (2%). Eighty percent of children had positive skin responses to one or more mycobacterial species. Among all children – and those with a BCG scar – there was no association between atopy or allergic symptoms and mycobacterial skin responses; among the few children without a BCG scar however those with positive mycobacterial responses were less likely to be atopic or to report allergic symptoms; these differences were not statistically significant.

Conclusions Our findings, in a population of BCG-immunized children, do not lend support to the suggestion that infection with atypical mycobacteria is protective against childhood allergic disease.

Keywords allergy, atopy, BCG, environmental mycobacteria, Greece

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Introduction

Protection against atopy, and from associated diseases such as hayfever or allergic asthma, may be distinct from each other and both may be related to microbial contact in early childhood. The mechanism of the effect(s), it is argued, could lie in the regulation of T-helper (Th) cell development and the appropriate predominance of Th1 and Th2 immune responses. In animal models, treatment with suspensions of killed environmental mycobacteria exerts a powerful immunoregulatory influence [1–3] and is effective in preventing or suppressing bronchial hyperresponsiveness. Such models are only illustrative of part of the chronic situation that may have developed over months or years in human patients. Practical benefits in the treatment of asthma and hayfever have been described following injections of heat-killed *Mycobacterium vaccae* [4, 5].

In a group of children living in rural parts of Crete, we have examined the cross-sectional relationships between atopy, allergic disease and skin test responses to four environmental mycobacteria encountered on the island. Children living in Crete are routinely vaccinated with BCG at the age of 6 years. We hypothesized that children with atopy and allergy would be less likely to exhibit positive skin test reactions to common mycobacterial antigens.

Methods

Mycobacterial reagents

We used mycobacterial skin test reagents from the range of 'New Tuberculin' [6]. These are prepared from individual species cultured on a non-antigenic medium (Sauton's agar), suspended in borate-buffered saline (pH 8.0) and treated with ultrasound to break open most bacilli. The sonicates are centrifuged, sterilized by filtration and their protein concentration is determined. With the exceptions of Nonchromogenicin and Vaccin (20 mcg/mL), solutions for skin-test use are diluted to a protein concentration of 2 mcg/mL.

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Pilot study

Three months before the survey we undertook a small study in a separate rural area to ascertain which of 20 environmental mycobacterial species (ten each fast- and slow-growing) infect Cretan children. Five groups of 10–11 boys each (a total of 53) aged 15 years were tested intradermally with four different species, two on the flexor aspect of each forearm. Boys were tested to avoid variable hormonal influences on responsiveness that have been recorded in earlier studies [7]. Skin responses, the diameters of induration, were read at 72 h. Details of the species and reagents tested and of the pilot results are summarized in Table 1. Based on these, four reagents were selected for the main survey. From each sub-genus we selected one species from among those inducing most sensitivity (to ascertain the overall prevalence of mycobacterial sensitization) and one from among those inducing least sensitivity (to identify children responsive to common mycobacterial antigens).

Main survey

We invited, through their parents, all schoolchildren between the ages of seven and 18 in four areas of Crete to take part in the survey. The areas were chosen to represent, in terms of topography, water supply, diet and type of farming, different rural lifestyles on the island.

In November 2001, we gave all children a questionnaire, which we asked their parents to complete. We also included a consent form for signature. Standardized questions enquiring into past and current symptoms of asthma and hayfever were translated from the questionnaire used in the International Study of Asthma and Allergy in Children (ISAAC). At the same time each child was invited to undergo skin prick tests (SPTs), in accordance with the ISAAC protocol, using a series of 10 common aeroallergens: grass pollen (Mediterranean mix), *Parietaria*, olive blossom, cat fur, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, goat epithelium, *Cladosporium*, cockroach and poultry, all purchased from ALK Abelló (Hungerford, UK). The negative and positive controls were saline and histamine, respectively. A SPT result was considered positive if it produced a mean weal diameter at least 3 mm greater than that produced by the saline control. In each of the schools over 80% of children took part in this phase of the study.

In January 2002, and no less than 8 weeks later for any child, we undertook intradermal skin tests with 0.1 mL of each of the four selected mycobacterial reagents (Aviumin C, Gordonin, Chelonin and Ranin I). As in the pilot study we read responses at 72 h, recording two orthogonal diameters of any induration. The intradermal skin tests were done and read without knowledge of questionnaire responses or of the results of the earlier skin prick testing. Of 854 eligible children, 806 (94%) completed mycobacterial skin testing in this way. At the time of testing, each child was examined for the scar of past BCG vaccination.

Prior approval for the study was obtained from the ethics committees of the Venizelion Hospital in Heraklion and Royal Brompton Hospital in London; and from the Ministry of Education in Greece. Both sets of skin tests were performed by experienced persons.

Statistical analyses

We devised two definitions of atopy: 'standard atopy' as a positive response to either house dust mite allergen, cat fur or any of the three pollens; and 'any atopy' as a positive response to any one or more of the 10 test allergens. We categorized mycobacterial skin test reactions by the mean diameter of any induration using either 3 mm (the minimum detectable response) or 10 mm diameters to distinguish positive responses. We further categorized children into those who responded to at least one reagent, those who responded to all four and those who responded to none [8, 9]. Comparisons were made between the prevalences of atopy or allergic disease among children with and without positive mycobacterial reactions, their statistical significance being determined, where appropriate, using χ^2 tests. Interactions between children with and without BCG scars were tested using logistic regression (Stata software).

Results

Pilot study

A summary of the pilot study results is shown in Table 1. Among the few children tested with each reagent a wide range of responses was uncovered. The prevalence of positive skin responses to the slow-growing species varied from over 80% with *M. intracellulare*, *M. kansasii*, *M. avium* type A and *M. scrofulaceum* to less than 30% with *M. gordonae*. Among fast-growing species only sensitivity to *M. chelonae* exceeded 80% whereas that to *M. flavescens*, *M. fortuitum* I and *M. gilvum* was less than 30%. As per our protocol we selected, for the main survey, four species: *M. gordonae*, *M. fortuitum*, *M. intracellulare* and *M. chelonae*.

Table 1. Pilot study results

Species	Reagent	n (%) positive	Mean (SD) diameter of induration, mm
Slow-growers			
<i>M. avium</i> A	Aviumin A	9 (82%)	9.7 (2.2)
<i>M. avium</i> B	Aviumin B	7 (70%)	8.6 (2.4)
<i>M. intracellulare</i>	Aviumin C	10 (91%)	9.4 (3.1)
<i>M. scrofulaceum</i>	Scrofulin	9 (82%)	9.4 (3.0)
<i>M. malmoensis</i>	Malmoensin	8 (73%)	8.4 (2.0)
<i>M. marinum</i>	Marinin	6 (60%)	9.0 (3.3)
<i>M. kansasii</i>	Kansasin	9 (90%)	8.0 (2.7)
<i>M. ulcerans</i>	Burulin	9 (45%)	6.4 (0.9)
<i>M. gordonae</i>	Gordonin	3 (27%)	6.7 (0.6)
<i>M. xenopi</i>	Xenopin	6 (60%)	8.0 (2.5)
Fast-growers			
<i>M. chelonae</i>	Chelonin	9 (90%)	10.1 (2.9)
<i>M. diernhoferi</i>	Diernhoferin	7 (64%)	8.3 (3.0)
<i>M. duvalii</i>	Duvalin	6 (60%)	6.2 (1.0)
<i>M. flavescens</i>	Flavescin	3 (27%)	6.7 (3.1)
<i>M. fortuitum</i> I	Ranin I	3 (27%)	6.0 (1.7)
<i>M. fortuitum</i> II	Ranin II	5 (50%)	6.0 (1.7)
<i>M. gilvum</i>	Gilvin	1 (9%)	7.0 (1.7)
<i>M. neoaurum</i>	Neoaurumin	5 (50%)	9.4 (3.8)
<i>M. nonchromogenicum</i>	Nonchromogenicin	8 (73%)	7.4 (1.5)
<i>M. vaccae</i>	Vaccin	8 (73%)	10.0 (2.2)

The species selected for the main survey are shown in bold type.

Of the 53 boys tested, 40 (75%) had scars of past BCG. Having a BCG scar tended to increase both the proportions responding to any intradermal tests and the mean positive reaction size although the differences were not statistically significant (data not shown).

Main survey

In the main survey, 23% of the children we tested had a positive prick response to one or more local allergens and were thus labelled atopic ('any atopy'); 146 (80%) of these, or 18% of the total, had positive responses to common allergens and were categorized as having 'standard atopy' (Table 2). Boys were twice as likely to be atopic as girls but there were no important differences by age. Children from the 'mountain area' were less often atopic but not significantly so.

Allergic symptoms – of hayfever or of wheeze ('ever') – were far less frequently reported but again more often for boys. Thirty (4%) children had one or more episodes of wheeze in the last year; of these nine (30%) were atopic. Thus the association between atopy and current wheeze was not strong (9/30 vs. 182/806; $P > 0.1$).

Table 3 displays the results of intradermal testing with the four mycobacterial species. Eighty percent of children had a positive response to one or more species, in almost all instances including *M. intracellulare* (Aviumin C). There were no sex differences but the oldest group, aged 15–18 years, were less likely to have a positive reaction to any of the tests ($P = 0.018$). Children living in either Lasithi or Anogia reacted significantly less frequently ($P < 0.01$) to individual reagents or to all four reagents than those who lived near Alikianos or in the mountain region. These findings are comparable with those in other eastern Mediterranean populations [10, 11].

We detected a scar of past BCG vaccination in 732 children. No scar was visible in 46; these did not differ in the distributions of their age, sex or region from those with a scar. For the remaining 28 children we were unsure; they have been left out of the following analyses. Children with a scar were more likely than those with no scar (82% vs. 59% $P < 0.001$)

to have positive reactions to the atypical reagents, a difference which was apparent for each species. This relationship was not confounded by age or region.

Among children with a BCG scar (and among the children as a whole) there were no important differences in atopic (or symptom) rates between those children who did or did not have positive responses to the mycobacterial skin tests (Table 4). In general, those with positive responses were more likely to be atopic than those without, although the differences were slight and not statistically significant. These findings were not affected by age but the differences were more pronounced among children living in Anogia. The same patterns were found when children with strongly positive intradermal reactions (those with indurations ≥ 10 mm) were compared with children whose reactions were negative.

Among the very small group of children in whom we could not see a BCG scar, the prevalence of atopy tended to be lower among those children with positive responses to the atypical reagents and no one with atopy reacted to all four new tuberculins (Table 4). None of these differences, however, reached statistical significance (all $P > 0.05$). All 13 children with pollen-sensitive hayfever had a BCG scar. Only one child without a scar was identified with atopic wheezing. These patterns, and their significance, were not materially altered by including those children who had an uncertain BCG scar status.

Discussion

Among a large and representative population of children living in four distinct areas of rural Crete we have found no clear association between measures of current atopy or associated allergic disease and intradermal skin reactions to four locally prevalent mycobacterial species. While it may be argued that the study was underpowered in respect of reported allergic symptoms (which were remarkably uncommon) the same seems unlikely for atopy, present in a fifth of the children we surveyed. The apparent dissociation between atopy and allergic disease that we found has been described in other parts of southeast Europe [12] but, as far as we are aware, remains unexplained.

We did however detect a modifying effect of prior BCG vaccination, although one that could readily have arisen by chance. Vaccination rates in Crete are very high, as reflected in our population; very few (c.2%) BCG recipients lack an obvious scar, and these are not known to differ immunologically from those with a scar. Among those in our population with no visible BCG scar, in contrast to those with a scar, children with positive responses to tests with the atypical reagents were less likely to be atopic or to have a history of allergic wheeze. That none of these differences was statistically significant may reflect the small size of this group. It could be argued that BCG vaccination – which probably enhances, but does not induce, the response to the many antigens common to all mycobacteria – masks the variation in skin response to atypical mycobacteria thus blurring the distinction between children who have and have not been infected with these organisms. If this is the case then our findings among unscarred children may reflect the true relationship between atypical infection and atopy. This could

Table 2. The prevalence of atopy and associated allergic symptoms by sex, age and area

	All	Atopy (standard)	Atopy (any)	Hayfever with positive SPT pollen	Atopic 'ever wheeze'
<i>n</i>	806	146 (18%)	182 (23%)	13 (2%)	41 (5%)
Male	394	99 (25%)	119 (30%)	10 (3%)	34 (9%)
Female	412	47 (11%)	63 (15%)	3 (1%)	7 (2%)
Age (years)					
7–12	513	87 (17%)	110 (21%)	7 (2%)	27 (5%)
13–14	162	34 (21%)	40 (25%)	4 (3%)	8 (5%)
15–18	130	25 (19%)	32 (25%)	2 (2%)	6 (5%)
Lasithi	126	25 (20%)	36 (29%)	0	3 (2%)
Anogia	295	56 (19%)	70 (24%)	4 (1%)	11 (4%)
Alikianos	281	52 (19%)	57 (20%)	7 (3%)	23 (9%)
Mountains	104	13 (13%)	19 (18%)	2 (2%)	4 (4%)

Figures are *n* (row%).

SPT, skin prick test.

Table 3. The prevalence of mycobacterial skin test results by sex, age and region

		Positive intradermal reaction (≥ 3 mm) to mycobacteria: <i>n</i> (%)						
	N	Any species	No species	All four species	<i>Mycobacterium gordonae</i>	<i>Mycobacterium fortuitum</i> I	<i>Mycobacterium intracellulare</i>	<i>Mycobacterium chelonae</i>
All	806	644 (80)	162 (20)	147 (18)	392 (49)	228 (28)	616 (76)	279 (35)
Male	394	319 (81)	75 (19)	67 (17)	173 (44)	111 (28)	311 (79)	145 (37)
Female	412	325 (79)	87 (21)	80 (19)	219 (53)	117 (28)	305 (74)	134 (33)
Age (years)								
7–12	513	419 (82)	94 (18)	99 (19)	264 (51)	150 (29)	405 (79)	172 (34)
13–14	162	132 (81)	30 (19)	32 (20)	87 (54)	48 (30)	123 (76)	63 (39)
15–18	130	92 (71)	38 (29)	16 (12)	41 (32)	30 (23)	87 (67)	44 (34)
Lasithi	126	98 (78)	28 (22)	17 (13)	53 (42)	28 (22)	91 (72)	47 (37)
Anogia	295	224 (76)	71 (24)	34 (11)	122 (41)	56 (19)	214 (72)	84 (28)
Alikianos	281	235 (84)	46 (16)	73 (26)	161 (57)	107 (38)	226 (80)	115 (41)
Mountains	104	87 (84)	17 (16)	23 (22)	56 (54)	37 (36)	85 (82)	33 (32)

Table 4. Relationships between measures of allergy and mycobacterial skin test responses

			Atopy (standard) <i>n</i> (%)	Atopy (any) <i>n</i> (%)	Hayfever with SPT+pollen <i>n</i> (%)	Atopic 'ever wheeze' <i>n</i> (%)
BCG scar Intradermal, mycobacterial reaction	Number		135	168	13	36
	Any species	+	115 (19)	143 (24)	13 (2)	32 (5)
		–	20 (15)	25 (19)	0	4 (3)
	All species	+	29 (21)	33 (24)	2 (1)	8 (6)
		–	106 (18)	135 (23)	11 (2)	28 (5)
	<i>M. gordonae</i>	+	71 (19)	85 (23)	9 (2)	17 (5)
		–	64 (18)	83 (23)	4 (1)	19 (5)
	<i>M. fortuitum</i> I	+	45 (21)	55 (26)	3 (1)	12 (6)
		–	90 (17)	113 (22)	10 (2)	24 (5)
	<i>M. intracellulare</i>	+	113 (20)	138 (24)	13 (2)	32 (6)
		–	22 (14)	30 (19)	0	4 (3)
	<i>M. chelonae</i>	+	54 (21)	66 (25)	6 (2)	15 (6)
		–	81 (17)	102 (22)	7 (2)	21 (5)
	Number		6	8	0	1
No BCG scar Intradermal, mycobacterial reaction	Any species	+	3 (11)	4 (15)	–	0
		–	3 (16)	4 (21)	–	1
	All species	+	0	0	–	0
		–	6 (15)	8 (21)	–	1
	<i>M. gordonae</i>	+	0	1 (8)	–	0
		–	6 (18)	7 (21)	–	1
	<i>M. fortuitum</i> I	+	0	0	–	0
		–	6 (16)	8 (22)	–	1
	<i>M. intracellulare</i>	+	3 (11)	4 (15)	–	0
		–	3 (15)	4 (20)	–	1
	<i>M. chelonae</i>	+	1 (8)	1 (8)	–	0
		–	5 (15)	7 (21)	–	1

SPT, skin prick test.

usefully be studied further in a BCG-naïve population with high rates of environmental mycobacterial infection.

Overall, children with a BCG scar had higher frequencies of atopy than those without a scar although the numbers in this latter category were very small and any differences were not statistically significant. Previous attempts to examine the effects of infection or vaccination with *M. tuberculosis* have provided conflicting results. Self-reported asthma and allergy were less prevalent among women (but not men) in Finland with a history of tuberculosis before the age of 20 years [13]; and an inverse relationship between delayed hypersensitivity responses to *M. tuberculosis* and atopy has been described in

a Japanese population [14]. This latter finding has not been corroborated in several other studies in different parts of the world [15–20]. Similarly the demonstration of an apparently protective effect of BCG vaccination on childhood atopy [21] has not been repeated elsewhere [22–24]. An ecological analysis [25] and the findings of reduced allergic disease among BCG vaccinated, non-native children living in Sweden [26] and Germany [27] can reasonably be explained by confounding. The children in Crete received BCG vaccination at 6 years of age by when many of them would probably have developed an atopic phenotype; we found no evidence of an increase in the prevalence of atopy with age. Thus it is not

possible from our study to discover whether BCG protects against the development of atopy.

It is useful to compare our findings with those of two other epidemiological studies of this subject. In a much larger survey of younger children (numbering almost 6500 and aged between 4 and 9 years) living in Sweden, Strannegard and colleagues [26] relied only on parental reporting of allergic symptoms. Compared with the children in Crete, 80% of whom responded to at least one mycobacterial species, far fewer (30%) of their population had a positive reaction to intradermal injection with two mycobacterial species. On the other hand, and in distinction to our population, allergic symptoms were not uncommon, being reported in 22% of children. Positive reactions to *M. avium* were slightly more common in 'allergic' children (37% vs. 32%) but there were no significant differences in responses to *M. scrofulaceum*. The authors concluded that there was no evidence to support the hypothesis that early (atypical) mycobacterial infection affects the balance of Th1/Th2 responses; and that the higher prevalence of positive reactions to one species was explicable by a reduced cell-mediated immunity in allergic children. Our findings, in a very different setting, are remarkably similar. Among a smaller population of Finnish children aged between 10 and 12 years and tested with extracts of *M. scrofulaceum* and *M. fortuitum*, just 6% and 4%, respectively, had responses of 5 mm or more. Those children reported, by their parents, to have allergic diseases, had on average smaller responses to the first of these species [28].

The interpretation of our findings depends critically on the assumption that skin test responses are a valid measure of previous mycobacterial infection. In fact a positive skin reaction depends not only on encounter (and infection) with a mycobacterium but also on the persistence of a dermal Th1 response and on human lymphocyte antigen haplotype [29]. In situations where mycobacteria are frequently met, as is probably the case in rural Crete, past sensitizations are kept boosted, whereas in situations where they are infrequently encountered memory cells persist but there may not be immediate recall of skin-test responsiveness. A prospective study might be expected more clearly to distinguish true rates of infection but only if immune responses in atopic children were determined very early in life.

If our findings, together with those of Strannegard et al., are taken at face value, then they appear to provide evidence against a role for childhood infection with atypical mycobacteria in the regulation and development of atopy, although the findings here are compatible with protection, among atopic children, from allergic disease. If anything, our results indicate that both Th1 and Th2 responses are increased among atopic children. An important caveat arises from the high rate of early BCG vaccination in this population and it remains possible that the effects of other mycobacteria might be important in communities where such immunization is delayed or not practiced.

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