

## Preliminary immunological studies in search of correlates of protective immunity carried out on some Iranian leprosy patients and their families

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**Summary** Multiple skin-testing, lymphocyte transformation tests and enzyme-linked immunosorbent assay of antibodies to mycobacterial antigens have been carried out on patients and their healthy children living in Baba Baghi Leprosy Sanatorium in Iran. The data reported shows a remarkable correlation between responses to *Mycobacterium leprae* and *M. vaccae* in all 3 test systems.

The percentage of positive responders to skin tests with Leprosin A amongst the children is higher than has previously been found and BCG has been shown to enhance the capacity of the individual to recognize *M. leprae* in this way. Finally, the majority of a small number of children considered to be protected from leprosy have been shown to possess lymphocytes that transform in the presence of *M. leprae* and *M. vaccae* antigens, but little antibody to *M. leprae* by the enzyme-linked immunosorbent assay. Of the 3 types of test assessed here only skin-testing appears to be of any value as a measure of protection, but whether even this will prove useful at the individual level is far from certain.

### Introduction

The purpose of this study was to evaluate 3 tests as correlates of protection from mycobacterial disease. It was carried out at Baba Baghi Leprosy Sanatorium near Tabriz in Iranian Azerbaijan. None of the children born and

brought up in the Sanatorium have developed leprosy in the 20 years that families have lived there, despite their frequent contact with new bacilliferous patients and a marked tendency amongst a few of the patients not to take their drugs. The majority of the children must therefore have protective immunity since most are in daily contact with bacilliferous cases of the disease. Since it is not possible to define a control group without protection we make a comparison between healthy children and the patients with whom they live. Of the 47 families living at Baba Baghi, 45 agreed to take part. Each person was skin tested and subsequently blood samples were obtained from individuals selected on the basis of skin-test results. These were used for lymphocyte transformation studies and enzyme-linked immunosorbent assay of antibodies.

## Materials and methods

### THE FAMILIES

Forty-two of the families consisted of leprosy parents and from 1 to 5 healthy children. One family consisted of the husband and 2 wives, all with leprosy and 3 healthy children. Two families consisted of a mother with leprosy, each with a single healthy child.

A grandfather lived with one of the families, an uncle with another family and an uncle and aunt with a third family. In each case these extra family members had leprosy. One of the healthy children was herself married to a leprosy patient.

Apart from a few children who were staying with relatives away from the Sanatorium or who had got married and left, and one child who went on holiday before the skin tests were read, every family member was skin tested. Some of the children had accompanied parents from their village when they first came to the Sanatorium, but most were born in the Sanatorium and had spent all their lives there. The total number of parents and other relatives studied was 94 and the total number of children studied was 113. (Additionally some skin-test results for other patients in the leprosarium have been added to Table 1.)

### SKIN TESTS

The reagents used were a range of new tuberculins prepared from live organisms grown on Sauton's agar, or, in the case of Leprosin A, from irradiated organisms harvested from armadillo tissues and freed from material of armadillo origin by the method of Draper (in Shepard *et al.*, 1980). In each case the harvested organisms were broken with ultrasound. The sonicates were sterilized by serial filtration, the protein content measured, and the final dilutions

**Table 1.** The results of skin-testing leprosy patients and their children with a series of new tuberculin

Reagent	Concentration	Patients + ve	Children + ve	Significance (Chi <sup>2</sup> )
Leprosin A	10 µg/ml	18/127	71/113	$P < 0.0001$
Vaccin	20 µg/ml	3/58	16/24	$P < 0.0001$
Nonchromogenicin	20 µg/ml	17/43	18/25	$P < 0.01$
Scrofulin	2 µg/ml	21/48	12/24	ns
Xenopin	2 µg/ml	2/31	5/25	ns
Rhodesin	2 µg/ml	0/19	6/25	ns
Marinin	2 µg/ml	28/40	19/25	ns
Diernhoferin	2 µg/ml	0/7	0/4	
Neoaurumin	2 µg/ml	0/6	3/8	
Gilvin	2 µg/ml	1/6	3/4	
Duvalin	2 µg/ml	0/6	2/4	
A*-in	2 µg/ml	10/19	4/5	
Aviumin C	2 µg/ml	2/7	1/5	
Gordonin	2 µg/ml	9/21	3/5	
Chitin	2 µg/ml	2/6	2/7	
Skinsnes'reagent	2 µg/ml	13/39	19/31	$P = 0.02$
ICRC reagents	2 µg/ml	5/12	10/15	ns
Kanasin	2 µg/ml	17/26	—	
Aviumin A	2 µg/ml	3/12	—	

In this table + ve refers to responses of 2 mm or more of induration to the skin-test reagents shown.

prepared in a tween containing borate buffer containing tween (Paul *et al.*, 1975). The concentrations used for skin testing were 20 µg/ml in the cases of Vaccin and Non-chromogenicin, 10 µg/ml in the case of Leprosin A, and 2 µg/ml for all the other reagents.

Every person was skin tested by the intradermal injection of 1 µg of Leprosin A and all but 11 of the youngest children (aged 1–3 years) were skin tested with 2 or 3 other reagents at the same time. Reactions were recorded as mean diameters of induration at 72 hours and reactions of 2 mm or more were considered positive. The list of reagents, numbers tested and numbers producing positive responses are shown in Table 1.

Ninety-two of the children had received BCG vaccination, a number of them on several occasions. This was confirmed by examination of their arms for scars. The distribution of the children by age, BCG status and response to Leprosin A is shown in Table 2.

#### IN VITRO TESTS

Blood samples were taken from 23 carefully selected children. Fourteen children who did not respond to skin test with Leprosin A agreed to be bled and in 9 cases there was a suitable Leprosin A-positive brother or sister who

**Table 2.** The effect of BCG and age on positivity to skin tests with Leprosin A

Age	BCG — ve Leprosin A + ve	BCG + ve Leprosin A + ve
<i>(A) Baba Baghi children</i>		
1	0/8	
2	0/6	3/4
3	0/3	1/3
4		3/6
5	0/1	2/5
6		0/4
7		2/3
		11/25 (44%)
8		2/2
9	0/1	5/7
10	1/1	10/11
11		10/12
12		7/8
		34/40 (85%)
13	0/1	7/7
14		6/6
15		6/8
16		4/4
17		1/1
18		
19		
20		1/1
		25/27 (93%)
	1/21 (4.8%)	70/92 (76%)
<i>(B) Children living in a town in the leprosy endemic area</i>		
12	4/9	13/24
13	3/14	9/27
14	2/9	16/26
	9/32 (28%)	38/77 (49%)

BCG + ve and — ve refer to vaccinated and nonvaccinated children respectively. Leprosin A + ve refers to children producing induration of 2 mm diameter or more to this reagent.

also agreed to be bled. As far as possible pairs from each family were of the same sex, roughly the same age and with the same BCG status. Five children were either single or had brothers or sisters too young to be bled for comparison. In every case the children came from families with at least one lepromatous parent. Blood samples were collected from 27 male leprosy patients (mainly lepromatous) for comparison with the children.

Lymphocyte transformation tests were carried out on cells separated with methyl cellulose in a system suitable for simple laboratory conditions as described by Rook and Stanford (in preparation). Cells were cultured in

RPM1 medium in the presence of 10% autologous serum. Leprosin A, Vaccin, Tuberculin and Nonchromogenicin were used as antigens at a final concentration of 20 µg/ml.

#### ENZYME-LINKED IMMUNOSORBENT ASSAY

This assay was performed as described by Voller *et al.* (1979) using peroxidase conjugated rabbit antisera to human immunoglobulin heavy chains (Dako). The antigens were the preparations used for skin-testing, diluted to 1 µg/ml and all sera were tested at a dilution of 1/800. The substrate was O-phenylene diamine. Data are presented as geometric means of absorbance values and statistical analysis was performed using log.-transformed absorbance values and Student's *t* test.

### Results

#### SKIN TESTS

The results of all skin tests carried out on the patients and their children are shown in Table 1 and the significance of differences between results for the 2 groups are also indicated ( $\text{Chi}^2$ ).

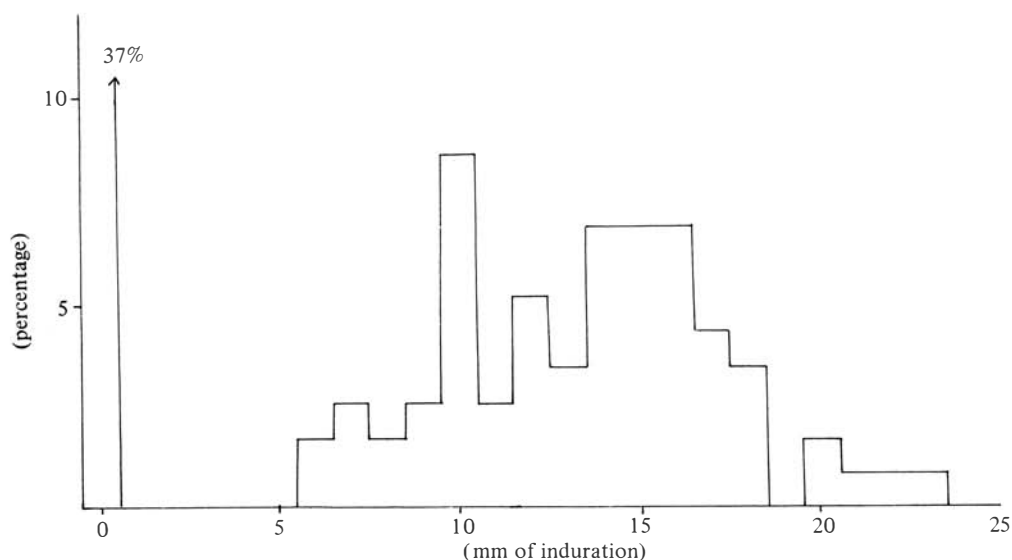
From Table 2 it can be seen that BCG has a considerable effect on development of Leprosin A positivity and so too does age. Thus of 21 children who had not received BCG only 1 was Leprosin A positive and of 92 BCG recipients 70 were positive. Among the BCG recipients, of the 25 aged 7 or less, 11 were Leprosin A positive, of the 40 aged 8–12, 34 were positive and of the 27 aged 13–20, 25 were positive. These results and the corresponding figures for Nonchromogenicin and Vaccin tests are shown together with the results obtained on the leprosy patients themselves in Table 3.

**Table 3.** The effect of age on response to 3 skin-test reagents in children who had received BCG. The results obtained in leprosy patients are shown for comparison

Patients	Positive responders to		
	Leprosin A 13/94 (14%)	Vaccin 1/17 (6%)	Nonchromogenicin 5/19 (26%)
BCG + ve, Children aged:			
2–7	11/25 (44%)	1/5 (20%)	1/1
8–12	34/40 (85%)	9/11 (82%)	7/12 (58%)
13–20	25/27 (93%)	6/7 (86%)	8/8 (100%)

*Mycobacterium nonchromogenicum* and *M. vaccae* were the two species shown to be related to the leprosy bacillus in skin-test studies in East Africa by Paul *et al.* (1975), and *M. vaccae* was the species most similar to *M. leprae* in some taxonomic studies (Stanford *et al.*, 1975; 1978). The sizes in millimetres

of responses of the children to Leprosin A are shown in Figure 1. Table 5 shows the mean positive reaction sizes to 5 reagents for patients and their BCG-vaccinated children. Statistical differences determined by Student's *t* test are indicated.



**Figure 1.** The percentages of children producing responses to Leprosin A expressed as millimetres of induration at 72 hours.

#### IN VITRO TESTS

The results of the lymphocyte transformation tests are shown in Figure 2 as ratios of uptake of radioactive thymidine by cultures in the presence of antigen and in its absence. The results for Leprosin A skin-test responders (LA + ve) and non-responders (LA - ve) are shown separately.

#### ENZYME-LINKED IMMUNOSORBENT ASSAY

Patients showed very significantly more IgG antibody to *M. leprae* and *M. vaccae* than either the Leprosin A - ve or the Leprosin A + ve children (Table 6). However, they did not have significantly more antibody than the Leprosin A + ve children, to *M. nonchromogenicum* or *M. tuberculosis*. There was also a tendency, not always significant, for Leprosin A + ve children to have *less* antibody to *M. leprae* and *M. vaccae* and *more* antibody to *M. nonchromogenicum* and *M. tuberculosis*, than the Leprosin A - ve children.

## Discussion

The overall percentage of positive responders to Leprosin A of 64% among the 113 children tested is the highest recorded so far with this reagent. Among the 92 children vaccinated with BCG 76% responded to Leprosin A. This association of Leprosin A positivity with BCG vaccination could be explained in one of two ways. Either there is marked cross-reaction between Leprosin and BCG or BCG enhances the individuals capacity to recognize *Mycobacterium leprae* met in the environment. Of these two alternatives the latter is likely to be the true explanation. This is shown in Table 2 by the effect of age on skin-test responses among the BCG recipients. Although not tabulated this same age effect among the BCG recipients is seen for each of the reagents to which a reasonable proportion of the children respond, and has been documented in previous studies (Paul *et al.*, 1975; Stanford *et al.*, 1976; Shield *et al.*, 1977; Stanford *et al.*, 1980). However, the effect is not seen with reagents prepared from mycobacterial species absent from the child's environment indicating that it is not cross-reactivity that increases with time after BCG vaccination. Recipients of the same BCG, aged 12–14 and still living in the region from which most of the patients originated, are much less responsive (49% positive) to Leprosin A than are the children reported here, (see the lower section of Table 2). Thus, in an environment where the leprosy bacillus is frequently encountered, BCG administration markedly enhances immunological recognition resulting in development of positivity to Leprosin A. That this is beneficial is suggested by the rarity of leprosy developing in these children. Thus to this extent positivity to skin-testing with Leprosin A is a measure of protection.

Unfortunately this is not likely to be the whole story, if it were then BCG vaccination might be expected to be effective against leprosy everywhere the disease is common. That other organisms are involved in the process of recognition of *M. leprae* was suggested by our earlier studies (Paul *et al.*, 1975) and has been supported by much as yet unpublished data from the field and from animal experiments.

Correlations between the results of testing with Leprosin A and with some of the other test reagents can be made. These are shown in Table 4 under two categories. The total correlations including failures to respond to both reagents and positive responses to both reagents. Positive correlations are also shown in which persons producing positive reactions to pairs of reagents are expressed as a percentage of all persons responding to at least one of the pair. Because too few persons were tested with some of the reagents for meaningful correlations to be derived the results have been pooled. 'Fast growers' include the results for Diernhoferin, Neoaurumin, Gilvin, Duvalin and Chitin; 'Slow growers' include A\*-in (made from an as yet unnamed species), Aviumin C and Gordonin. The reagents prepared from 9 of Professor Skinsnes' strains grown from leprosy patients fall into 2 clusters, a group of 6 reagents showing little correlation

**Table 4.** The relationships between skin-test responses to Leprosin A and to the other reagents tested. See text for an explanation of the correlations shown

'Other' reagent	Leprosin A + ve	'Other' reagent + ve	Correlations	
			Total	Positive
Vaccin	19/41	17/41	39/41 (95%)	17/19 (89%)
Nonchromogenicin	22/44	22/44	33/44 (75%)	17/27 (63%)
Marinin	23/51	37/51	34/51 (67%)	22/38 (58%)
ICRC 1204	15/27	13/27	21/27 (78%)	10/18 (56%)
ICRC 1203	15/27	9/27	20/27 (74%)	8/16 (50%)
Skinsnes × 6	26/92	8/92	64/92 (70%)	2/32 (6%)
Skinsnes × 3	20/38	14/38	32/38 (84%)	14/20 (70%)
Fast growers	16/46	11/46	37/46 (80%)	9/18 (50%)
Slow growers	9/36	14/36	29/36 (81%)	7/16 (44%)
Scrofulin	19/41	16/41	30/41 (73%)	12/23 (52%)
Rhodesin	22/44	6/44	27/44 (61%)	6/22 (27%)
Xenopin	22/44	6/44	25/44 (57%)	4/24 (17%)

with Leprosin A and a group of 3 reagents showing much better correlation. Much the best correlation is shown by *M. vaccae* which appears to have some special relationship with *M. leprae*. Of the 17 individuals producing positive responses to Vaccin all produced positive responses to Leprosin A, although there were 2 responders to Leprosin A who did not respond to Vaccin. Thus positivity to Vaccin may also be a measure of protection from infection. Other species also show a correlation with Leprosin A, although to a much smaller extent. It is interesting to note that reagents prepared from 3 of Professor Skinsnes' isolates from leprosy tissues show 70% of positive correlation with Leprosin A. More results are needed with these strains to confirm this particularly since the concentration of reagents used may not have been optimal. The nature of the relationships reflected by correlations of skin-test results is not clear and again simple cross-reactivity does not appear to be the explanation. In unpublished experiments on mice, Vaccin positivity can be shown to enhance the animals capacity to develop positive reactions to Leprosin A after challenge with small doses of leprosy bacilli. Observations in animals of a relationship between *M. leprae* and *M. vaccae* have also been made by Watson *et al.* (1979). Among groups of people, mean positive reaction sizes may be of importance. Thus the children tend to produce larger-sized responses to Leprosin A than do the patients (Table 5), and the same is true of Vaccin. The reverse is the case for Marinin prepared from *M. marinum*, an organism apparently frequently met by patients and children. The results of lymphocyte transformation tests with Leprosin A (Figure 2) do not correlate with the skin-test results. Although they show some differences between the children and lepromatous patients. Of the 23 children tested, 3 show markedly greater transformation than the others. Transformation in the presence of Vaccin produced results remarkably similar to those with Leprosin A and the highest results occur in the same 3 children.



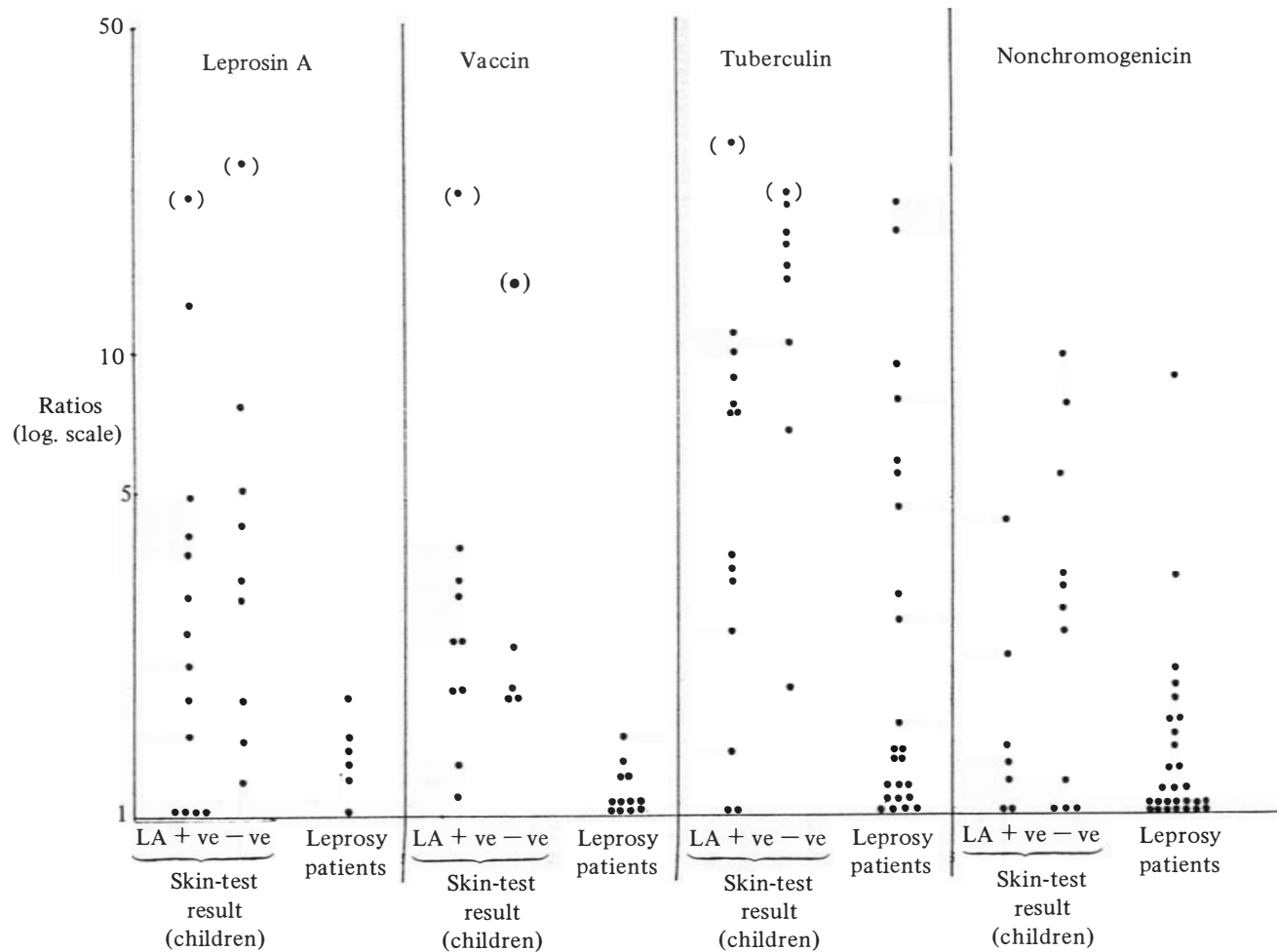


Figure 2. Lymphocyte transformation test results.

**Table 5.** Mean positive reaction sizes (2 mm or more induration at 72 hours) of leprosy patients and their BCG-vaccinated children to 5 skin-test reagents. Statistical significance for the different reaction sizes between the patients and the children has been calculated by Student's t test. Numbers of positive reactors are shown in parentheses

Reagent	Mean positive reaction sizes		Significance (t test)
	Patients	BCG + ve children	
Leprosin A	11.67 mm (18)	13.53 mm (70)	$P < 0.08$
Vaccin	5.3 mm (7)	8.33 mm (16)	$P < 0.03$
Nonchromogenicin	10.12 mm (17)	10.0 mm (16)	ns
Scrofulin	11.8 mm (21)	11.8 mm (12)	ns
Marinin	16.3 mm (26)	12.7 mm (19)	$P < 0.004$

BCG + ve refers to children who have been vaccinated with BCG.

Nonchromogenicin and Tuberculin induced a considerable amount of transformation amongst some of the lepromatous patients and did not discriminate between any of the groups. The 3 children producing the highest results with Leprosin A and Vaccin were not tested with Nonchromogenicin, but with Tuberculin, 2 of the 3 produced the highest results of their groups. Our results do not indicate that lymphocyte transformation tests of the type described here would have any value as a measure of protection. However, recent studies (Bahr *et al.*, in preparation) suggest that more detailed analysis of the regulation of such lymphoproliferative responses may provide valuable information.

**Table 6.** The results of ELISA of immunoglobulin to the antigens of 4 mycobacterial species. The results are expressed as absorbance values

	<i>M. leprae</i>	<i>M. vaccae</i>	<i>M. nonchromogenicin</i>	<i>M. tuberculosis</i>
Patients	0.29	1.93	0.189	0.42
	$P < 0.0001$	$P < 0.0001$	$P = 0.004$	
Leprosin A + ve children	0.07	0.59	0.24	0.33
	$P = 0.1$		$P = 0.0002$	$P = 0.002$
Leprosin A - ve children	0.107	0.65	0.087	0.18

The ELISA results (Table 6) again pointed to a relationship between *M. leprae* and *M. vaccae*, and also indicated that the development of a positive leprosin A response is not associated with an increased level of antibody against these organisms. Skin-test positivity may even be associated with lower levels of antibody to *M. leprae* and related species, in which case measurement of antibody to selected antigenic components may prove to be of value in the future.

The lack of development of overt leprosy lesions in these children with

considerable exposure to *M. leprae* over their entire lifetimes provides evidence of their lack of susceptibility to the disease. Thus the observations made are likely to reflect the immunological status of the protected individual. Probably the 2 or 3 children differing from the others have recently undergone or are presently experiencing some mycobacterial challenge. Whether they will successfully overcome it and their immunological parameters return to that of their fellows, and whether their fellows will in turn experience similar immunological disturbances we hope to discover by retesting the same individuals over a number of years.

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