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Lepromin conversion induced by a 'sub-unit' vaccine from ICRC bacilli

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A sonicate of ICRC bacilli yielded on gel permeation HPLC a major high molecular weight (apparent mol wt 1000K) glycolipoprotein, PP-1, and a number of low molecular weight peaks (the pooled fraction named PP-II). PP-I induced Mitsuda-type skin reaction in healthy subjects. When administered as a vaccine, PP-I also induced lepromin conversion in lepromatous leprosy patients and their lepromin negative healthy house-hold contacts. In view of the importance of lepromin-test as an index of host immunity, the possibility of using PP-I in the preparation of a 'sub-unit', anti-leprosy vaccine has been discussed.

The ICRC anti-leprosy vaccine, that is currently undergoing large scale clinical trials in India, brings about lepromin (Mitsuda) conversion in lepromatous (LL) patients and their lepromin negative health house-hold contacts. Some vaccinated patients also exhibit 'up-grading' of tissue lesions[1–4].

Intradermal administration of an integral antigen of *Mycobacterium leprae* (lepromin) results in local induration that peaks between 3-4 wk (Mitsuda reaction). This reaction, which is characteristic of *M. leprae*, is observed in 80-90 per cent of healthy people[5]. The test has been used by Shepard and Guinto[6] as a marker for immunological identification of mouse foot-pad isolates as *M. leprae*. Although its precise mechanism is still not fully understood, clinical, epidemiological and laboratory evidence indicates that the Mitsuda reaction correlates well with host-defence against *M. leprae*[7–10]. Any mycobacterium that induces Mitsuda-type reaction is likely to be antigenically closely related to *M. leprae*, particularly in components that are responsible for the skin reaction. Earlier studies from our laboratory have shown that, an integral antigen of ICRC ($ICRC_{in}$) gives Mitsuda-type skin reaction comparable to that induced by Mitsuda lepromin, except that the response induced by the former tends to be of a larger magnitude[1].

With a view to identify its Mitsuda-inducing component/s, we have fractionated sonicate of ICRC bacilli using gel permeation high performance liquid chromatography (HPLC). In the present study, we have tested different HPLC-fractions in monkeys which

exhibit a spectrum of lepromin reaction similar to that seen in man[11,12]. It was observed that, of the various HPLC fractions tested in the animals, only the high molecular weight fraction (PP-I), which is a glycolipoprotein, induced the Mitsuda-type of reaction. For these reasons, human studies were carried out only with PP-I and we further demonstrated that PP-I, in antigenic doses, gives Mitsuda-type reaction in healthy subjects. Also, a vaccine containing PP-I, (henceforth called the 'sub-unit' vaccine) was found to induce lepromin conversion in a number of LL patients and their lepromin negative healthy house-hold contacts.

Material & Methods

Source of the ICRC bacilli : Strain C-44 of the ICRC bacilli used in this study, was isolated from a biopsy from a LL patient in 1969. The organisms are now in the 116th passage. Cultivation is routinely done in a medium essentially consisting of Dubos base with amino acids and vitamins as in Dulbecco's minimum essential medium, 10 per cent human AB blood group serum, supplemented with minerals, (ferric ammonium citrate, $MgSO_4$, $CuSO_4$, $CaCl_2$ and $ZnSO_4$). 0.5 ml of 10 per cent Tween 80 per litre is added as the dispersing agent. The medium also contains 0.5 per cent tetradecane and 5 per cent dimethylsulphoxide as recommended by Kato[13]. The cultures were harvested between 13-15 days, and the mycobacteria washed extensively with normal saline.

HPLC : The organisms were sonicated for 2 h (optimal time determined by preliminary studies) in a Biosonik III sonicator (Brownwill Scientific Co., Rochester, NY) at 80 W intensity at 4°C to obtain maximum yield of extractable proteins. The sonicate was centrifuged at 50,000 rpm in a Kontron Ultracentrifuge, for 1 h at 4°C, and the supernatant filtered through a 0.22 μ millipore filter to remove any particulate substances. The supernatant was aliquoted, lyophilised and stored at—20°C. Fractionation of the sonicate was carried out on HPLC (Waters Associates, Milford). The buffer system consisted of 0.02 *M* Tris acetate *p*H 7.2, and the flow rate was 1 ml/min. Optical density was recorded at 280 nm. The soluble proteins were separated on a Protein Pak 300 SW gel permeation column (exclusion limit 400 K). The sonicate separated into a major high molecular weight fraction, and a number of relatively low molecular weight fractions. The high molecular weight fraction, which eluted in void volume at the retention time of 5.21 min and accounted for 70 per cent of the proteins of the sonicate, was collected separately and called PP-I. The rest of the fractions were pooled, and the combined fraction called PP-II. The fractions PP-I and PP-II were dialysed against water, lyophilized, and stored at—20°C. Before use the lyophilized fractions were reconstituted in a suitable volume of normal saline and sterilized by filtration using millipore filter. The reconstituted PP-I was administered intradermally, on the volar surface of the forearm, irrespective of whether it was used as the antigen or vaccine. The proteins were estimated by Hartree's method[14], and concentrations of 200 μg and 1 mg per ml were used as the antigen and vaccine, respectively. Only 0.1 ml was injected per person. The dose used for vaccination was arbitrarily taken as five times in excess of the antigen dose. Lipids were estimated by the method of Saito and Sato[15]. For estimation of carbohydrates, method of Dubois *et el*[16] was used.

Studies in monkeys : Three adult lepromin

negative male Langur monkeys (*Presbytis entellus*), which inhabit north India, were administered ICRC anti-leprosy vaccine as described elsewhere[11]. This resulted in lepromin conversion in all the animals. The animals were administered 50 μg each of the sonicate, PP-I and PP-II intradermally. The sonicate was given in the upper third of the right forearm, while PP-I and PP-II were administered in the upper and middle thirds of the left forearm respectively. The local reaction was measured at 48 h and, subsequently, at weekly intervals up to 3 wk, and erythema/induration recorded.

Healthy volunteers and house-hold contacts : The study in the healthy volunteers and contacts was conducted in Malwani, a suburb of Bombay, with a population of about 63,000; using the Seth G.S. Medical College's primary community health center at Malwani as the base. The residents of Malwani belong to the economically lower middle or poor class. In this suburb there were a total of 691 leprosy patients, giving a prevalence rate of 10.6/1000. There was no segregation of patients.

Volunteers : Locale reponse to antigenic challenge of PP-I (20 μg/0.1 ml/person) was studied in 10 healthy male adults aged between 25 and 40 yr, belonging to the lower middle class. Four weeks after administration of PP-I, 8 of the 10 subjects were subjected to the lepromin test.

Contacts : One hundred healthy house-hold contacts of active LL patients, were tested for lepromin (Mitsuda) reactivity. Of the 16 who were lepromin negative, the 'sub-unit' vaccine was administered to 10 (group I). The other 6, who received saline, served as controls (group II). Lepromin test was repeated at 10 wk in both the groups.

Patients : Studies in patients were conducted on resident inactive LL patients in Acworth Leprosy Hospital, Bombay. All patients were under continuous treatment with DDS, for periods varying between 5-25 yr. A total of 27 patients including 7 females, between 16 and 60 yr of age, participated in the study. Diagnosis was based on the clinical and bacteriological features, at the time of admission. Twenty one patients received multidrug treatment (rifamycin, clofazmine and DDS) initially for 2-3 yr followed by DDS alone. The other 6 patients were on DDS monotherapy for 15-25 yr. At the time of study, clinical features mainly included wrinkled areas over the body, loss of eyebrows (partial or complete), depressed bridge of the nose and residual deformities over hands and feet. Patients were clinically inactive and bacteriologically negative as confirmed by repeated bacteriological examination. All were lepromin negative. No sex related differences were observed.

Before initiation of the study, lepromin test was performed on all patients, who were then divided into three groups *viz.*, groups III, IV and V of 10, 9 and 8 patients respectively. Each patient in groups III and IV received the 'sub-unit' vaccine (100 μg PP-I/person), and the test was repeated 3 months later. Following the repeat test, patients in group IV received a booster (100 μg PP-I/person). Four months later, the test was repeated for the second time in both the groups. Patients in group V, who served as controls, were given only saline which was used as the vaccine vehicle. Lepromin test was repeated in them at 3 and 7 months, corresponding to the intervals of the other two groups.

The human studies were cleared by the ethical committee of the Institute and in-

formed consent of the vaccinees was obtained.

Lepromin (Mitsuda) test : The test was performed using 0.1 ml of Mitsuda lepromin, containing 4×10^7 bacilli/ml. The antigen was given on the volar surface of the forearm. Upper third of left and right forearms were used for per-vaccination and first repeat post-vaccination tests, respectively. When lepromin test was performed for the third time in groups III, IV and V, the middle third of the left forearm was used. Lepromin was obtained through the kind courtesy of Dr W.F. Kirchheimer, National Hansen's Disease Center, Carville, Louisiana, USA, with the assistance of the World Health Organization. The local response was recorded at 3 wk. Induration of 3 mm and above denoted the positive response.

Biopsies : Representative punch biopsies of the (*i*) local reaction induced by PP-I when used in antigenic doses in healthy contacts (group I) and (ii) third lepromin tests in group III and IV patients were obtained and fixed in neutral buffered formalin. 5 μ paraffin sections were stained with haematoxylin and eosin.

Results

HPLC : The HPLC profile of the sonicate of ICRC bacilli is shown in Fig. 1. The high molecular weight PP-I, that accounts for around 70 per cent of the protein, eluted in the void volume. In order to determine its molecular size, some samples of PP-I were further passed through u Bondagel E 1000 (exclusion limit 2000 K). It eluted as a single peak after Blue dextran and just preceeded IgM which has a molecular weight of 900,000. For these reasons, an apparent mol wt of 1000 K was assigned to PP-I.

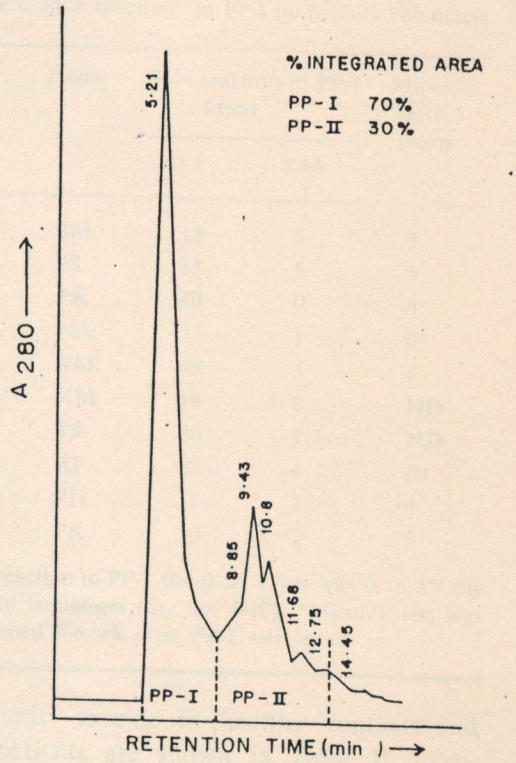

Fig. 1. HPLC profile of the ICRC sonicate. It shows one major (PP-I) and four minor peaks (pooled fraction PP-II). Retention time is in min. The figure also depicts percentage integrated area of the two fractions (PP-I and PP-II) as recorded by the HPLC monitor.

The amounts of carbohydrates and lipids in PP-I were 47.25 $\pm$ 2.70 and 25.00 $\pm$ 5.33 per 100 units of proteins respectively (results expressed as the mean $\pm$ SD) These values are based on analyses of 6 samples collected from different batches of ICRC bacilli.

Studies in monkeys : A typical reaction pattern following administration of the sonicate, PP-I and PP-II in the monkeys indicates that only the sonicate and PP-I induce the late (Mitsuda-type) reaction (Fig. 2).

Studies in healthy volunteers : Response

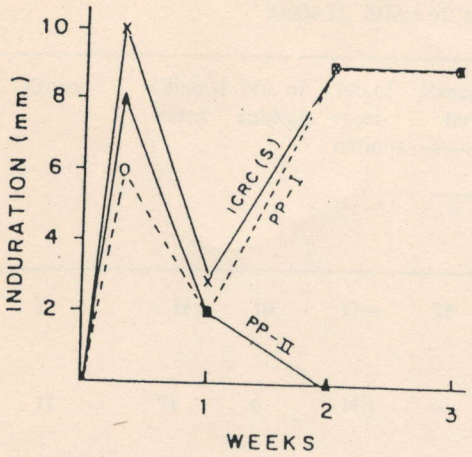

Fig. 2. Typical skin responses to intradermal administration of the sonicate and its PP-I and PP-II fractions in monkeys. Mitsuda-type (late) response is elicited only by the sonicate and PP-I. [ICRC(s) : sonicate].

to antigenic challenge in healthy volunteers: Local response to administration of PP-I in healthy volunteers is shown in Table I. At 6 h, all individuals developed erythema, which was maximum at 48 h (only the latter readings are shown). At the end of the 1st wk, the site of injection appeared almost normal. However, in the following days a small induration, which peaked at 3 wk, was observed. In general, induration tended to be small in size. There was marked variation in the response. In 2 subjects (sr. nos. 3 and 6) no induration was observed. On the other hand, induration of 3 mm and above was observed in 4 subjects (sr. nos. 1, 2, 5, 8) in whom the Mitsuda test was also positive. A representative biopsy (Fig. 3) of the local response to PP-I showed chronic inflammation with lymphocytes, foamy macrophages, a few epithelioid cells, and ill-formed giant cells.

Response to vaccine in contacts and patients: Effects of administration of the 'sub-unit' vaccine in healthy contacts and LL patients are shown in Table II. Progressively increasing erythema/induration was observed at the site of administration of the 'sub-unit' vaccine. The local reaction was similar in both the contacts as well as in the patients. Maximal reaction was seen at 48 h. Three weeks later most of the erythema had disappeared and a mean induration of 5-7.5 mm was seen in different vaccinated groups. No ulceration was observed at the vaccination site. A few vaccinated subjects developed mild fever for 1-2 days but no systemic untoward effects were seen. No exaggerated reaction was observed after the booster in group IV patients (Table II).

Lepromin (Mitsuda) conversion rates were high (80 %) in the vaccinated healthy house-hold lepromin negative contacts

Table I. Skin reaction to PP-I in healthy volunteers

Sr. no.	Name	Skin reaction to PP-I (mm)		Mitsuda test* (mm)
		48 h	3 wk	
1.	SM	15	3	8
2.	SS	35	5	5
3.	PK	40	0	4
4.	NA	22	1	0
5.	RM	45	5	6
6.	KM	18	0	ND
7.	TS	30	2	ND
8.	RP	38	4	10
9.	PN	18	2	4
10.	PK	30	2	6

Skin reaction to PP-I (mm) at 1 wk was 0 in all the healthy volunteers (Sr. no. 1-10). *Mitsuda test was performed 5-6 wk after PP-I was tested

Table II. Effect of 'sub-unit' vaccine on Mitsuda reaction

Group	Clinical status	No. of subjects	No.of vacci-nations	Reaction at the vaccination site (erythema/induration mm*)				Lepromin (Mitsuda) test (No. of +ve subjects)		
				1st		2nd		Initial	Repeat (Lepromin converion)	
				E	L	E	L		I	II
I	H	10	One	28	5.0	—	—	Nil M (0.4) R (0-1)	8 (80%) M (4) R (3-6)	—
II	H	6	Nil	—	—	—	—	Nil M (0.6) R (0-1)	Nil (0.5) (0-1)	—
III	P	10	One	26	7.5	—	—	0	1 (10%) (4)**	1 (10%) (6.5)**
IV	P	9	Two	24	6.3	20	7.0	0	Nil	3 (33%) M (5.2) R (4-6.5)
V	P	8	Nil	—	—	—	—	0	Nil	Nil

*Only the mean values for the group are shown. E, early (48 h); H, healthy house-hold contacts; M, mean induration in mm; L, late (3 wk); P, LL patients. R, ranges. Under the column 'Repeat', ranges of only positive responses are depicted. **Induration in mm

(group I). Only one out of 19 vaccinated LL patients (group III and IV) exhibited conversion 3 months after vaccination. However, 3 (33 %) of the 9 (group IV) were converted after the booster. In 2 patients, lepromin conversion was confirmed by biopsy which showed lymphocyte-rich granuloma containing epithelioid and giant cells (Fig. 4). No lepromin conversion was seen in the control groups (groups II and V; Table II).

Discussion

The sonicate of ICRC bacilli, organisms that have been used in preparation of an anti-leprosy vaccine, yields one major and four minor peaks on HPLC. The major peak (PP-I), accounts for approximately 70 per cent of the proteins of the antigen and contains a high molecular weight glycolipo-protein. PP-I not only induces Mitsuda-type local reaction in healthy subjects but, when given in sufficient quantity, as a vaccine, it also induces lepromin conversion both in LL patients and their lepromin negative house-hold contacts.

One of the significant features of this study is the use of monkeys to screen bacillary fractions for their ability to induce Mitsuda-type skin reaction. For such studies, it should be ensured that experimental animals are lepromin positive, so that a negative response, if any, is not merely due to their

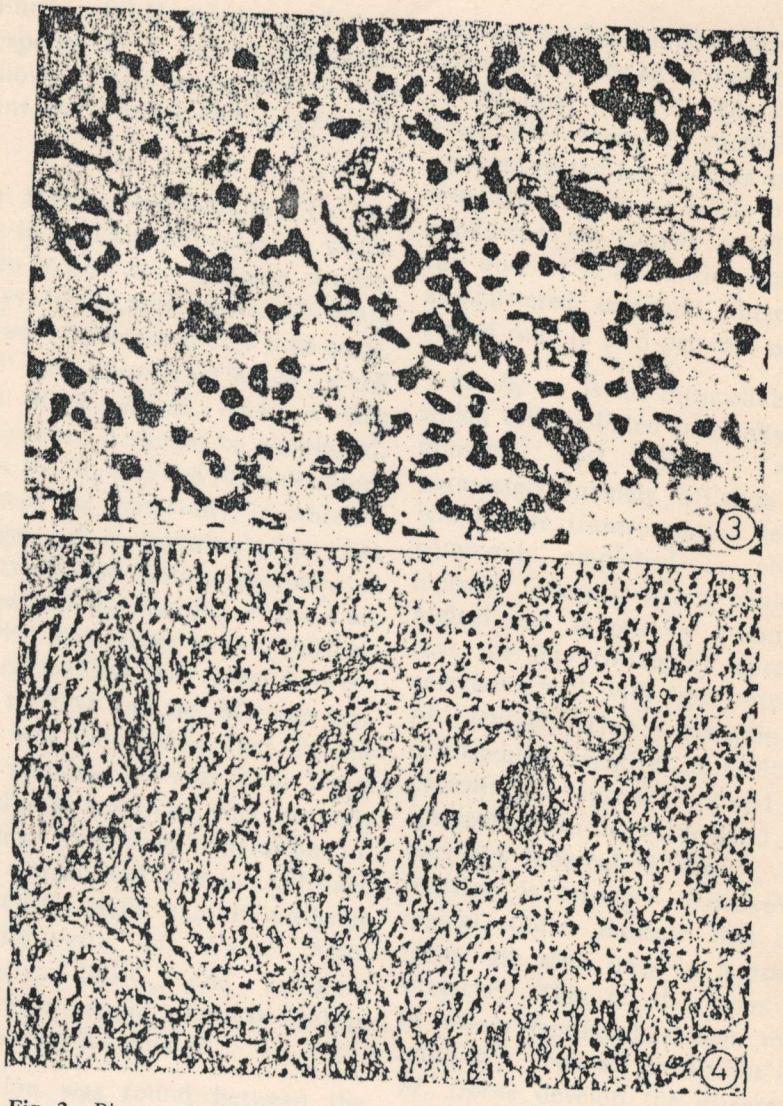

Fig. 3. Biopsy of the 3 wk local response to PP-I in a healthy volunteer showing chronic inflammation with lymphocytes, foamy macrophages a few epithelioid cells and ill-formed giant cells (H and E × 600). **Fig. 4.** Biopsy of the site of lepromin reaction of a lepromin positive group IV patient showing granulomatous inflammation rich in lymphocytes, epithelioid and giant cells. (H and E × 220).

inability to mount the skin response. Considerable variation is observed in the spectrum of lepromin reaction in unvaccinated monkeys. This problem was overcome by first vaccinating the animals before they were used to test various fractions. All the 3 vaccinated animals gave a p ve lepromin reaction. When the sonicate, PP-I and PP-II were tested in these vaccinated animals, only the first two could induce he Mitsuda-type

reaction. Further, PP-II also did not induce the late response in 3 human volunteers (data not shown). The results indicate that PP-I contains the Mitsuda-inducing components.

This was further substantiated by the observations in healthy volunteers, in whom the PP-I also induce skin response similar, in many ways, to Mitsuda reaction in man, particularly with reference to the time scale. Reitan *et al*[17] have shown that MLW_1, a cell wall antigen of *M. leprae*, elicits a skin reaction. However, it should be mentioned, that, whereas MLW_1 elicits a 48 h reaction, our's is perhaps the first study, in which a soluble antigen of a mycobacterium has induced Mitsuda-type local response. It must be stated that the intensity of the 3 wk reaction to PP-I was rather weak. Further, no definite correlation was seen between the response to PP-I and the Mitsuda test. However, it was interesting that in the subjects with response of more than 3 mm to PP-I, lepromin reaction was strongly positive. Using lymphocyte migration inhibition test, Gangal and Khanolkar[18] have shown that ICRC bacilli exhibit antigenic cross reactivity with *M. leprae*. Also, integral antigens, $ICRC_{in}$ and lepromin induce comparable responses both in healthy subjects and leprosy patients[1,19].

No correlation was found between the duration of treatment and lepromin conversion in the patients. Sample size in the study is rather small to draw any definitive conclusion regarding the stability of the immune conversion induced by PP-I. However, in the group III patient who exhibited lepromin conversion, it was stable for a minimum period of 7 months. Although very high lepromin conversion rates were observed in healthy contacts, only 33 per cent of the LL patients exhibit immune conversion. Lower conversion in the latter, may simply be related to the dose of PP-I. On the other hand, even after repeated vaccination, only 40 per cent of the LL patients exhibited a positive skin response to whole bacilli in studies conducted by Convit *et al*[20]. When the full dose of ICRC anti-leprosy vaccine was administered, lepromin conversion was observed only in 53.5 per cent of LL patients[1].

M. leprae is an intracellular parasite, in which cell mediated immunity (CMI) is the dominant host defence. Two laboratory parameters, namely Mitsuda reaction and lymphocyte transformation test (LTT), are commonly employed to assess CMI in leprosy. There is strong clinical, epidemiological and laboratory evidence indicating that the late (Mitsuda) lepromin reaction correlates well with host resistance against *M. leprae*[5]. Thus, lepromin reaction is consistently negative in multibacillary LL patients who represent one end of the leprosy spectrum. On the other hand, in the paucibacillary tuberculoid variety, the lepromin reaction is strongly positive[7]. Epidemiological studies indicate that multibacillary forms are seen only in lepromin negative subjects[8]. This is also true of monkeys in whom leprosy has been experimentally induced[12]. Not all armadillos infected with *M. leprae* develop the disease. According to Job *et al*[10] Mitsuda positive armadillos are relatively resistant to the disease. On the other hand, according to Bjune[21], LTT, using *M. leprae* antigens, is not a good indicator of protective immunity. It correlates well with cellular hypersensitivity. Mitsuda test appears to be a better, if not the only index, of 'protective' immunity.

Attempts are being made at the global levels, to develop a 'sub-unit' anti-leprosy

vaccine. Using monoclonal antibodies to screen the secreted products, Young *et al*[22] have demonstrated 5 proteins on cloning *M. leprae* genome in *Escherichia coli*. In order to use these gene products as 'sub-unit' vaccines, it will be necessary to first identify the 'protective' antigen(s). We have followed an alternative strategy of isolating an immunogenic fraction, PP-I, from sonicated ICRC bacilli, used in preparation of anti-leprosy vaccine. Chemically, PP-I is a glycolipoprotein with the relative proportions of protein: carbohydrate: lipid being 58:27:15. The exact chemical nature of the Mitsuda-inducing moieties of the PP-I is under investigation. Chemical nature of PP-I indicates that it is possibly associated with the cell wall, the first cellular component that comes in contact with immune cells. It should, therefore, logically play a dominant role in inducing immunity. Surface components/antigens are increasingly being used in preparation of effective vaccines[23–25].

Several groups are now investigating fundamental aspects of immunology of leprosy[26–28]. However, laboratory correlates of protective immunity are not yet fully defined. At present, amongst the available laboratory parameters, the Mitsuda test perhaps exhibits the best correlation with host immunity. Interestingly, much of the anti-leprosy vaccine development, so far, has been based on skin-test reactivity[29]. Any organism or its component that induces Mitsuda conversion, specially in LL patients who exhibit a persistent negative response, even after years of drug theraphy[30], offers a high possibility of containing 'protective' antigen/s and should be a candidate for vaccine preparation. In view of this, further clinical studies are being carried out on PP-I.

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