Vaccination of Human Volunteers with Heat-killed *M. leprae*: Local Responses in Relation to the Interpretation of the Lepromin Reaction[1]

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In 1919, Mitsuda reported that some human subjects, including leprosy patients, injected with a suspension made from lepromatous tissue, developed a local nodular reaction ([9]). Since then, successive improvements, such as the one made possible by the availability of armadillo-derived *Mycobacterium leprae* ([6]), have resulted in a more refined Mitsuda test. It is now known as the lepromin test and is widely used for both classification and prognosis in leprosy.

There are essentially two responses to the lepromin test: an early response known as the Fernandez reaction, which is measured between 24 and 72 hours, and the late response known as the Mitsuda reaction, which is measured between 3 and 4 weeks, after testing ([3]). The Fernandez reaction is very similar to the tuberculin reaction in that it measures the subject's delayed-type hypersensitivity to soluble bacillary antigens ([8, 13]). The Mitsuda reaction, on the other hand, is thought to be an induced hypersensitivity granuloma ([7, 15]), also in response to soluble bacillary antigens. The longer onset time for the Mitsuda response is believed to be due to the need for the processing and ultimate release of these antigens.

As Mitsuda had originally observed, it is now well established that patients with leprosy respond in a characteristic way to the lepromin test: patients at the tuberculoid end of the leprosy spectrum have positive Fernandez and Mitsuda responses; lepromatous patients do not respond to lepromin ([7]). Mitsuda also observed that most normal people responded positively to his test ([9]). This has been confirmed in normal individuals living in nonendemic countries or in countries with low endemicity who have been found to be Mitsuda positive but Fernandez negative ([1, 7, 11, 14]). It is this latter finding which has made the interpretation of the lepromin test difficult.

Recently, a trial of an armadillo-derived, heat-killed *M. leprae* vaccine was conducted among healthy volunteers living in a nonendemic country ([3–5]). This study permitted us to examine their early and late responses to lepromin, their skin-test responses to a soluble *M. leprae* antigenic preparation (MLSA) and to PPD, their *in vitro* lymphocyte transformation responses to various antigens and, in addition, the relationships among these responses.

MATERIALS AND METHODS

Study design. Four groups of individuals, between 23 and 28 years of age, were given 1.5×10^7, 5×10^7, 1.5×10^8, and 5×10^8 killed *M. leprae* intradermally according to a protocol which has been described in detail elsewhere ([3]). Briefly, the volunteers were skin tested with PPD (purified protein derivative) 1 month before vaccination, after which individuals were assigned to groups so that the range of the PPD responses was evenly distributed among the groups. A month after this initial PPD skin testing, the first group of volunteers was skin tested with coded antigens, PPD, and a soluble preparation derived from *M. leprae* (MLSA). Seventy-two hours later, the volunteers were vaccinated. The skin testing was repeated 3 months after vaccination. Blood samples for

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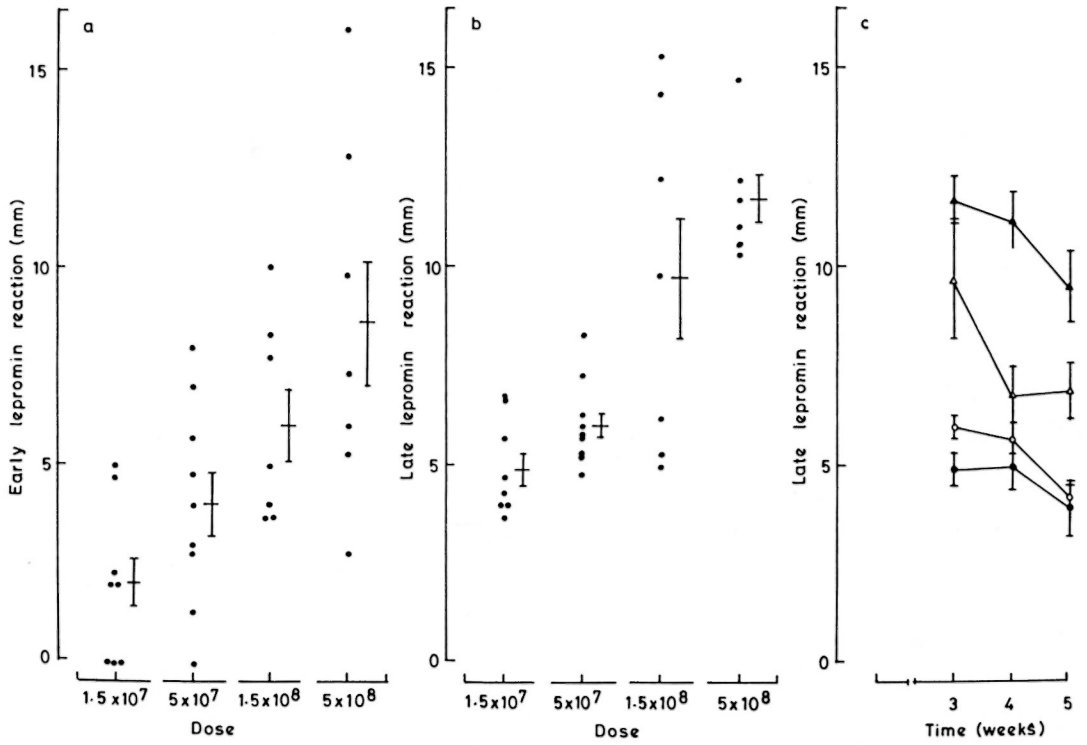

FIG. 1. Dose response of the early and late lepromin reactions and the kinetics of the late reaction. Four groups of individuals were vaccinated with 1.5×10^7, 5×10^7, 1.5×10^8, and 5×10^8 heat-killed, armadillo-derived *M. leprae.*

Fig. 1a shows the early lepromin response and Fig. 1b shows the late lepromin response in these four groups. In Fig. 1a and 1b each dot represents the response of a single individual; results are expressed as the mean response in each group. In Fig. 1c, results are expressed as the mean response of the groups (Group 1 = ●, Group 2 = ○, Group 3 = △, Group 4 = ▲) at 3, 4, and 5 weeks; vertical bars represent the S.E.M.

serum antibody testing and *in vitro* lymphocyte transformation were taken 3 days before vaccination and then 1 month, 3 months, 6 months, and 12 months after vaccination.

Antigens. The heat-killed, armadillo-derived *M. leprae* vaccine and the skin-test antigens, PPD, and MLSA were provided by IMMLEP (courtesy of R. J. W. Rees) as described elsewhere ([3]). For the lymphocyte transformation test (LTT), both the soluble *M. leprae* antigen (batch CD 45) and the human *M. leprae* were kindly provided by Dr. R. J. W. Rees. PPD was obtained from the Serum Institute, Copenhagen, Denmark.

Skin testing. The volunteers were skin tested with the soluble antigen on the volar surface of the forearm. The tests were read at 48 hr and 72 hr by measuring the horizontal and vertical diameters of the induration reaction. The skin-test responses were finally expressed as the mean of these two diameters taken at 72 hr ([3]).

Local reactions. Using a standard grid (an equilateral triangle of 3 cm), the vaccine was injected intradermally into three sites on the deltoid region. The local reactions to the vaccine were measured at 72 hr for the early lepromin reactions and at 3 weeks, 4 weeks, and 5 weeks after vaccination for the late lepromin reactions. The horizontal and vertical diameters of infiltration and ulceration were recorded, and the mean of the six measurements from the three sites has been expressed as the local reaction to the vaccine.

Lymphocyte transformation test. Peripheral blood mononuclear cells (PBMC) from a single vaccinee, taken at various intervals, were removed from storage in liquid nitrogen, thawed, and assessed for viability ([5]). The PBMC were cultured at a concentration of 10^5 cells per well in 96-well U-bottom trays in the presence of antigens, added in

THE TABLE. *Intersite variation of local reactions (mm induration) to vaccine.*

Subjects	Vaccine injection site	Time of reaction reading			
		72 hr	21 days	28 days	35 days
ATT	1	10.0	14.0 U[a]	12.0 U	11.5 U
	2	9.0	17.0 U	12.5 U	12.0 U
	3	9.5	13.0 U	13.5 U	11.5 U
HK	1	6.5	N.A.[b]	N.A.	9.0 U
	2	5.0			8.5 U
	3	7.5			9.5 U
ER	1	4.0	11.0 U	12.5 U	9.5 U
	2	0	9.0 U	13.0 U	8.5 U
	3	4.5	11.0 U	13.0 U	11.0 U
AF	1	12.0	11.5 U	9.5 U	7.0 U
	2	11.0	10.0 U	8.5 U	6.0 U
	3	15.5	11.5 U	9.0 U	5.0 U
NHH	1	17.5 U	11.5 U	12.0 U	10.0 U
	2	15.5 U	12.0 U	12.5 U	10.5 U
	3	15.0 U	13.0 U	12.5 U	12.0 U
KS	1	5.5 U	12.5 U	10.0 U	11.5 U
	2	5.5 U	12.0 U	11.5 U	10.5 U
	3	5.0 U	12.5 U	11.0 U	11.0 U
OB	1	6.5	10.5	9.5 U	7.5 U
	2	8.0	10.5	9.5 U	8.5 U
	3	7.5	10.5	9.5 U	7.5 U

[a] U = ulceration.
[b] N.A. = not available.

triplicate. The trays were incubated for 6 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. On day 6, the cultures were pulsed with 0.045 MBq ^{3}H-thymidine (specific activity = 185×10^3 MBq/mmole) for 4 hr, after which they were harvested with a Skatron harvester (Norway). The radioactivity incorporated was determined by liquid scintillation spectroscopy. The results were expressed as the median value of counts per minute (cpm) of each triplicate.

Statistics. Pearson's method was used to calculate the correlation between two sets of observations, except where LTT data were involved, in which case the Spearman method was used to calculate correlations.

RESULTS

Intersite variation of reactions to vaccine. At all doses tested the intersite variation in reaction size or ulceration was very small (representative results of Group 4 subjects are given in The Table). This would indicate that the precision of the lepromin test was not significantly increased by giving multiple tests simultaneously.

Dose response and kinetics of early and late lepromin reactions. There was a clear dose-response relationship for both the early (Fig. 1a) and the late reactions (Fig. 1b). Furthermore, the lepromin response did not reach a plateau within the range of doses tested. The kinetics of the late response (Fig. 1c) showed the 3-week responses to be as strong as or stronger than the 4-week reactions. In subsequent analysis the 3-week reaction was used to express the Mitsuda response.

Pre-vaccination reactions in relation to early and late lepromin reactions. As shown in Figures 2a and 2b, no apparent association was found among the pre-vaccination skin-test response to MLSA and the early (corr. = 0.22, $p = 0.25$) and late (corr. = 0.39, $p = 0.21$) lepromin reactions. However, a positive correlation between the pre-vaccination MLSA skin-test response and early lepromin reaction was found for Group 4 (corr. = 0.80, $0.05 < p < 0.02$). A lack of association was also observed between pre-vaccination PPD skin-test responses and the early (corr. = 0.25, $p = 0.19$) and late (corr. = 0.22, $p = 0.24$) lepromin reactions (Fig. 3, a and b). There was also no association between the pre-vaccination LTT response to MLSA and the early (Fig. 4a; corr. = 0.46, $p = 0.11$) and late (Fig. 4b; corr. = 0.35, $p = 0.26$) lepromin reactions. Similarly, there was no association between the pre-vaccination LTT response to PPD and the early lepromin reaction (Fig. 5a; corr. = 0.46, $p = 0.11$), but there was a borderline correlation between the pre-vaccination LTT response to PPD and the late lepromin reaction (Fig. 5b; corr. = 0.57, $p = 0.04$).

Post-vaccination responses in relation to late lepromin reaction. As shown in Figure 6, there is an association between the post-vaccination skin-test response to MLSA and the late lepromin reaction (corr. = 0.60, $p < 0.0002$). The pattern revealed that a weakly positive late lepromin reaction was associated with heterogenous skin-test responses, while a strong late lepromin reaction (>10 mm or ulceration) was always associated with a positive MLSA response. There was no correlation between the 1-month post-vaccination LTT response to MLSA and the late lepromin reaction (Fig. 7; corr. = 0.37, $p = 0.23$).

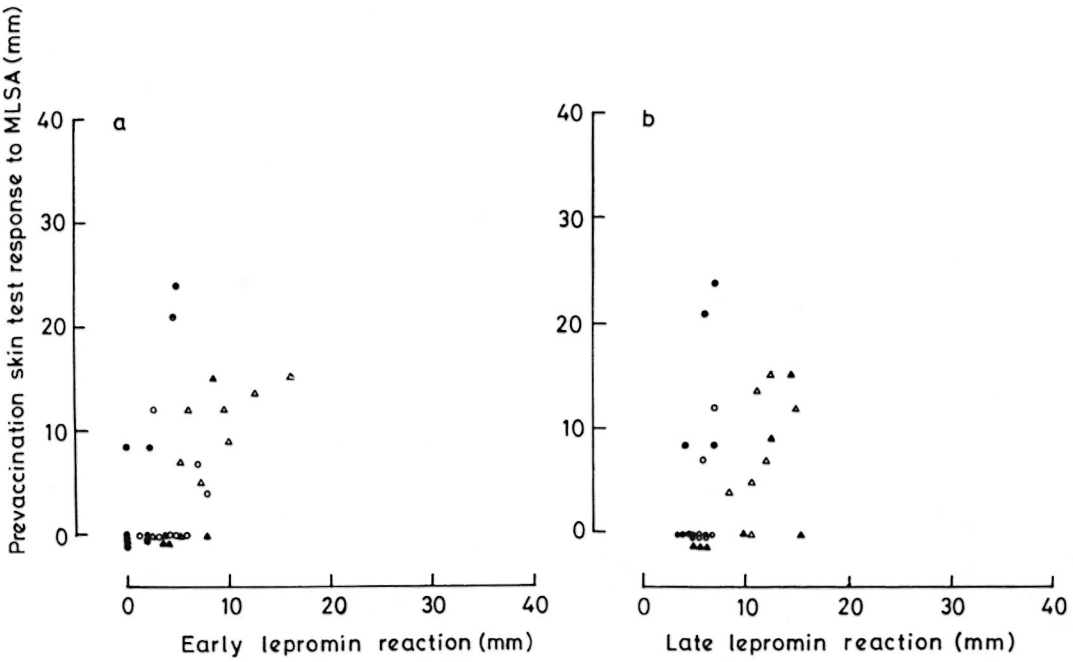

FIG. 2. Relationship between the early and late lepromin reactions and the pre-vaccination skin-test response to MLSA. Four groups of individuals were vaccinated with 1.5×10^7 (●), 5×10^7 (○), 1.5×10^8 (▲), and 5×10^8 (△) bacilli.

Fig. 2a shows the correlation between the early lepromin reaction and the pre-vaccination skin test to MLSA (corr. = 0.39, p = 0.21). Fig. 2b shows the correlation between the late lepromin reaction and the pre-vaccination skin test to MLSA (corr. = 0.22, p = 0.25).

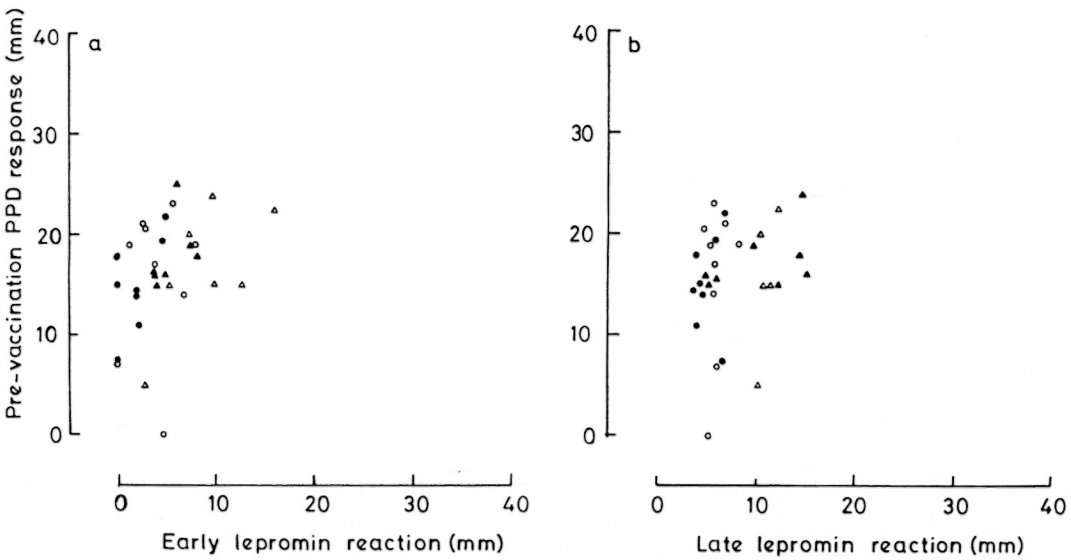

FIG. 3. Relationship between the early and late lepromin reactions and the pre-vaccination skin-test response to PPD. Four groups of individuals were vaccinated with 1.5×10^7 (●), 5×10^7 (○), 1.5×10^8 (▲), and 5×10^8 (△) bacilli.

Fig. 3a shows the correlation between the early lepromin reaction and the pre-vaccination skin-test response to PPD (corr. = 0.25, p = 0.19). Fig. 3b shows the correlation between the late lepromin reaction and the pre-vaccination skin-test response to PPD (corr. = 0.22, p = 0.24).

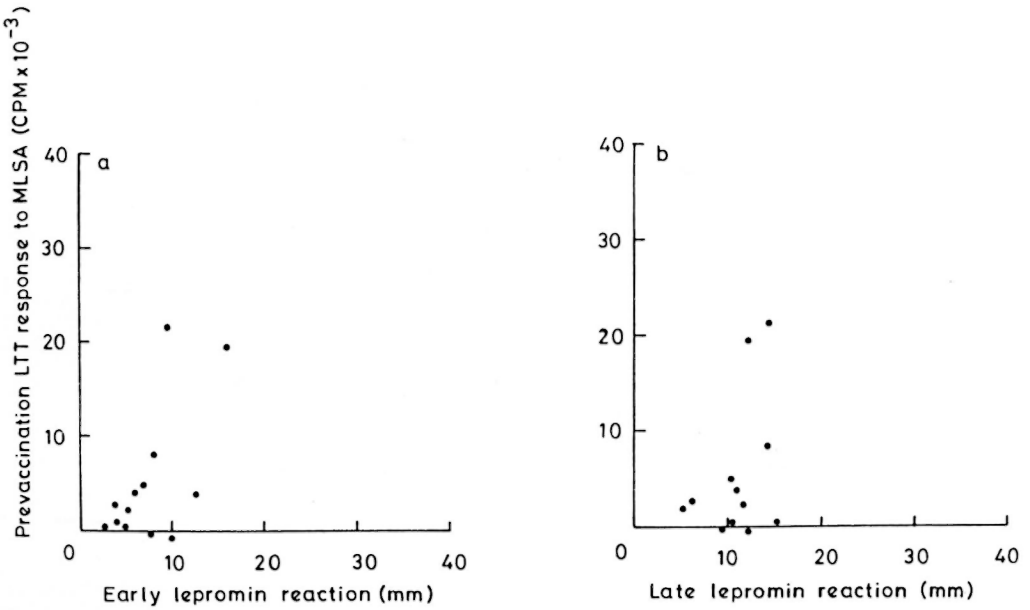

FIG. 4. Relationship between the early and late lepromin reactions and pre-vaccination LTT response to MLSA. Two groups of individuals were vaccinated with 1.5×10^8 and 5×10^8 bacilli.

Fig. 4a shows the correlation between the early lepromin reaction and the pre-vaccination LTT response to MLSA (corr. = 0.46, p = 0.11). Fig. 4b shows the correlation between the late lepromin reaction and the pre-vaccination LTT response to MLSA (corr. = 0.33, p = 0.30).

Early lepromin response in relation to late lepromin response. There is a good association between the early and the late lepromin responses (corr. = 0.70, $p < 0.001$) when they are compared with respect to reaction size (Fig. 8). However, it must be noted that the cut-off point for a positive early reaction (>10 mm) is different from the cut-off point for a positive late lepromin reaction (>3 mm). Thus, while 19 out of 22 subjects gave negative early lepromin responses, all subjects (22/22) gave positive late lepromin responses.

DISCUSSION

The uncommonly high number of Mitsuda responders among healthy individuals living in nonendemic countries has been somewhat puzzling. Since most populations have been vaccinated with BCG, Mitsuda reactivity could be attributed to antigenic crossreactivity between BCG and *M. leprae.* However, our study, in which there was no correlation between the subject's pre-vaccination PPD reactivity and the early and late lepromin responses, does not support this proposition. The recent work of Ponninghaus and Fine ([12]) also does not support this proposition. They found that a population that had been vaccinated with BCG had relatively high positive conversion rates to *M. leprae* skin-test antigens 90 days after vaccination, but that these conversion rates waned when the same population was skin tested 9 months after vaccination.

The other view of the lepromin test is that it is a form of vaccination. Our study would appear to confirm this view. Normal, healthy individuals injected with doses of vaccine ranging from 1.5×10^7 bacilli to 5×10^8 bacilli gave positive Mitsuda responses. Thus, the lepromin test would appear to be unsuitable as a diagnostic test for leprosy, as Mitsuda pointed out in his early study, because of its inability to distinguish between infected and noninfected individuals.

The present study also reveals the critical importance of the dose of lepromin used. Both the early and the late lepromin responses do not reach a plateau within the range of doses tested. Furthermore, a significant skin-test conversion was achieved only with the three highest doses of vaccine, i.e., 5×10^7, 1.5×10^8, and 5×10^8 bacilli ([3]). Mitsuda responses, on the other

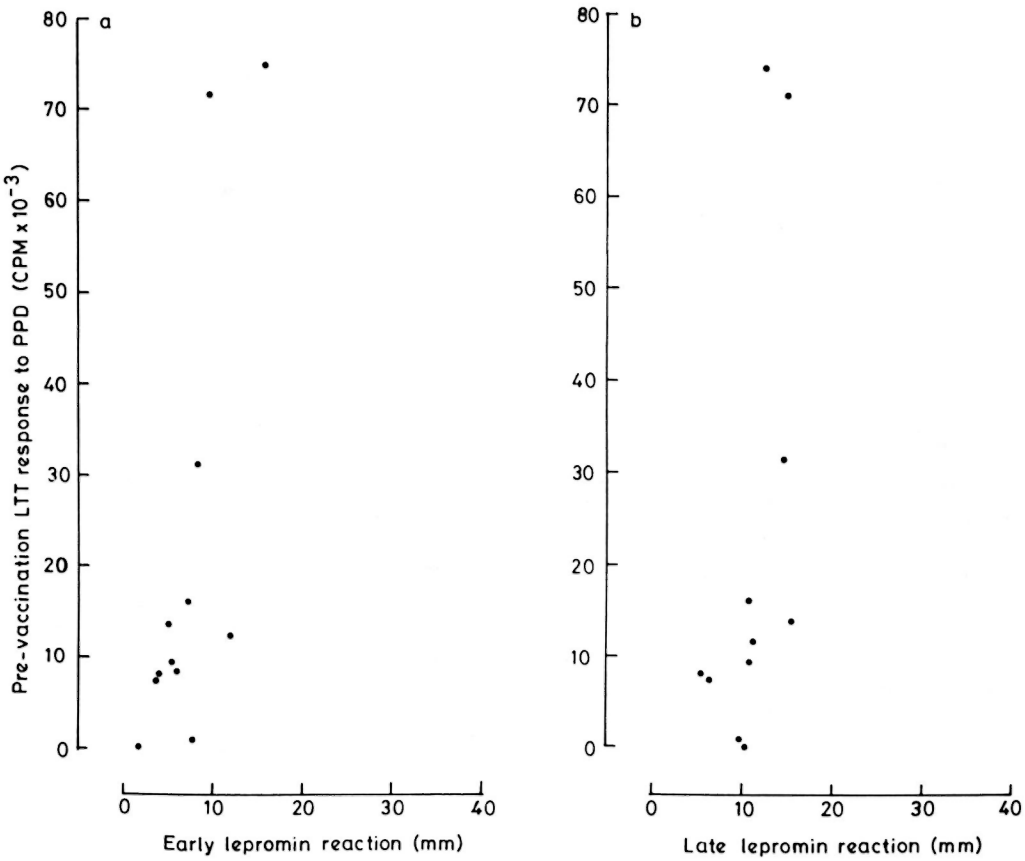

FIG. 5. Relationship between the early and late lepromin reactions and the pre-vaccination LTT response to PPD. Two groups of individuals were vaccinated with 1.5×10^8 and 5×10^8 bacilli.

Fig. 5a shows the correlation between the early lepromin reaction and the pre-vaccination LTT response to PPD (corr. = 0.46, p = 0.11). Fig. 5b shows the correlation between the late lepromin reaction and the pre-vaccination response to PPD (corr. = 0.57, p = 0.04).

hand, are elicited with doses ranging between 4×10^6 and 1.6×10^7 bacilli ([15]). In this lower range of doses a positive conversion is not always achieved. Therefore, if the lepromin test is being used to decide if a person is capable of mounting a cell-mediated response against *M. leprae,* the dose of *M. leprae* used would have to lie between 5×10^7 and 5×10^8 bacilli.

The Mitsuda reaction has also been studied extensively in the contacts of leprosy patients. In this situation, it is used to measure exposure and to identify susceptible individuals. In the dose range that is being used, especially if the lepromin is armadillo-derived, the lepromin test may be negative because of the low dosage used rather than the inability of the individual to respond.

It is generally believed that the Fernandez reaction is a measure of previous sensitization with *M. leprae.* However, since we found no association between the pre-vaccination skin tests to MLSA and the early lepromin reaction, our results do not appear to support this belief. We did find an association, however, between these two parameters at the highest dose of lepromin used—5×10^8 bacilli—which accords with our previous speculation that the amount of a soluble antigen in lepromin preparations may be too variable to give rise to a consistent delayed-type hypersensitivity (DTH) response ([11]). Under these circumstances, the explanation for the close correlation observed, quantitatively, between the early and late lepromin reactions in this and an earlier study ([11]) is not immediately obvious. Such an explanation may have to await the availability of pure antigenic preparations.

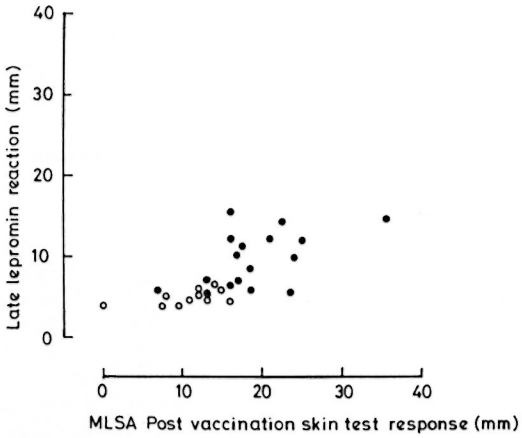

FIG. 6. Relationship between the late lepromin reaction and the MLSA post-vaccination skin-test response. Four groups of individuals were vaccinated with 1.5×10^7, 5×10^7, 1.5×10^8, and 5×10^8 heat-killed, armadillo-derived *M. leprae*. The figure shows the correlation between the late lepromin reaction and the MLSA post-vaccination skin-test response (corr. = 0.64, p = 0.0002). Ulceration = (●); no ulceration = (○).

The present trend in the identification of important *M. leprae* antigens ([10]) will soon lead to the availability of more specific and standardized antigens for skin testing and diagnosis in leprosy. However, until then the critical importance of the type of antigen used, the dose of the antigen, and the time at which the response is assessed should be recognized in the interpretation of skin tests in leprosy.

SUMMARY

The early (Fernandez) and late (Mitsuda) lepromin reactions were closely examined in a group of healthy, BCG-vaccinated individuals who were given four doses of a heat-killed, armadillo-derived vaccine, i.e., 1.5×10^7, 5×10^7, 1.5×10^8, and 5×10^8 bacilli. There was a clear dose-response relationship for both the early and late reactions with no leveling of the responses within the range of doses examined. While the early response was negative in most of the volunteers, the late response was positive in all of the volunteers. No association was found between the early lepromin test and the pre-vaccination skin test to PPD. There was also no association between the early lepromin test and the pre-vaccination skin test response to a soluble *Mycobacterium leprae* antigenic preparation (MLSA) in general, but there was a good correlation between these two parameters at the highest vaccine dose. The late lepromin response showed no association with either the pre-vaccination or post-vaccination skin test response to PPD. However, there was a significant correlation between the late lepromin response and the post-vaccination skin test response to MLSA. In general, no association could be found between the *in vivo* skin tests and the *in vitro* lymphocyte transformation test (LTT). Thus, the lepromin test is essentially a vaccination which elicits a specific response to *M. leprae* antigens provided that the dose of armadillo lepromin given is higher than 5×10^7. Therefore, it is unsuitable as a diagnostic test for leprosy. The variability of the early lepromin responses, especially in the lower range of lepromin doses, precludes its use as a measure of previous sensitization. However, the more consistent late lepromin response

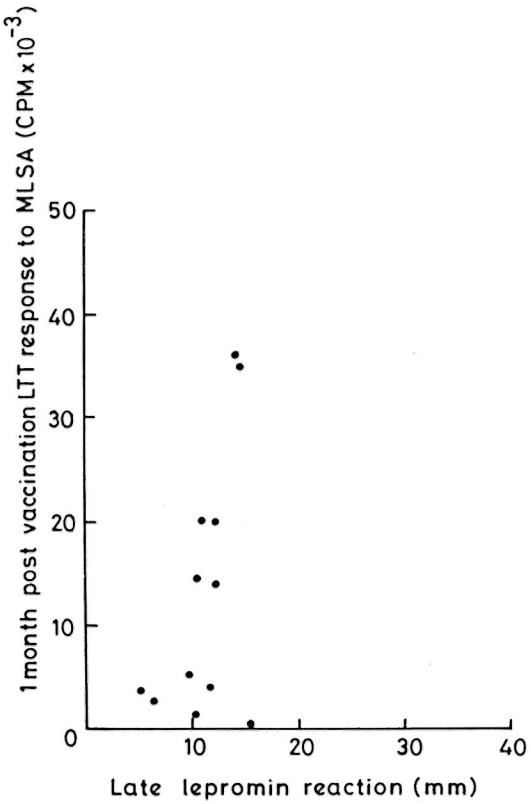

FIG. 7. Relationship between the late lepromin reaction and the 1-month post-vaccination LTT response to MLSA (corr. = 0.37, p = 0.23). Data shown are for the two groups which received 1.5×10^8 and 5×10^8 bacilli.

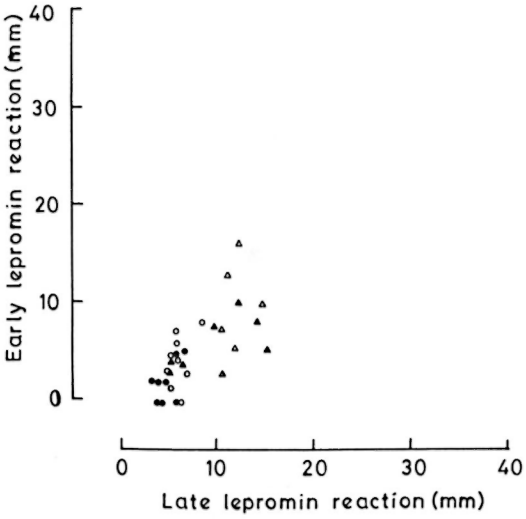

FIG. 8. Relationship between the late lepromin reaction and the early lepromin reaction (corr. = 0.68, p = 0.0001). Data shown are for the four groups of individuals who were vaccinated with 1.5×10^7 (●), 5×10^7 (○), 1.5×10^8 (▲), and 5×10^8 (△) heat-killed, armadillo-derived *M. leprae.*

could still serve to identify susceptible individuals.

RESUMEN

Se examinaron detalladamente las reacciones temprana (Fernández) y tardía (Mitsuda) de la lepromina en un grupo de individuos sanos vacunados con BCG los cuales recibieron 4 dosis de una vacuna derivada de armadillos conteniendo 1.5×10^7, 5×10^7, 1.5×10^8, y 5×10^8 bacilos. Hubo una clara relación de dosis-respuesta tanto para las reacciones tempranas como para las tardías, sin nivelación de las respuestas dentro del rango de dosis examinadas. Mientras que la respuesta temprana fue negativa en la mayoría de los voluntarios, la respuesta tardía fue positiva en todos ellos. No se encontró asociación entre la reacción temprana a la lepromina y la reacción dérmica prevacunación en respuesta a una preparación antigénica soluble derivada del *Mycobacterium leprae* (ASML) pero hubo una buena correlación entre estos dos parámetros a la dosis más alta de la vacuna. La respuesta tardía a la lepromina no mostró asociación con la respuesta al PPD ni en la prevacunación ni en la post-vacunación. Sin embargo, hubo una correlación significativa entre la respuesta tardía a la lepromina y la respuesta dérmica al ASML post-vacunación. En general, no se pudo encontrar asociación alguna entre las pruebas dérmicas *in vivo* y las pruebas de transformación de linfocitos *in vitro.* Así, la prueba de la lepromina es esencialmente una vacunación que induce una respuesta específica a los antígenos del *M. leprae* siempre y cuando la dosis de bacilos en la lepromina de armadillos sea mayor de 5×10^7. Por lo tanto, la prueba no es adecuada para el diagnóstico de la lepra. La variabilidad de la respuesta temprana a la lepromina, especialmente con las dosis bajas de la misma, excluye se uso como medida de sensibilización previa, sin embargo, la respuesta tardía a la lepromina (la cual es más consistente) aún podría servir para identificar a los individuos susceptibles.

RÉSUMÉ

On a examiné de manière attentive les réactions précoces (Fernandez) et tardives (Mitsuda) à la lépromine, dans un groupe d'individus sains vaccinés par le BCG, auquel on a administré du vaccin dérivé du tatou et tué par la chaleur, aux doses respectives de $1{,}5 \times 10^7$, 5×10^7, $1{,}5 \times 10^8$, et 5×10^8 bacilles. On a observé une relation dose-effet tout à fait nette, à la fois pour la réaction précoce et pour la réaction tardive, les réponses étant bien identifiables d'après la dose dans la gamme des doses qui ont été envisagées. Alors que la réponse précoce était négative chez la plupart des volontaires, la réaction tardive était positive chez tous. Aucune association n'a été relevée entre l'épreuve précoce à la lépromine et l'épreuve cutanée au PPD effectuées avant la vaccination. On n'a pas davantage enregistré d'association entre les résultats fournis avant vaccination par l'épreuve précoce à la lépromine et par l'épreuve cutanée avant la vaccination avec une préparation antigénique soluble de *Mycobacterium leprae* (MLSA); on a cependant observé une bonne corrélation entre ces deux paramètres pour les doses de vaccin les plus élevées. La réponse tardive à la lépromine n'a montré aucune association avec les épreuves cutanées au PPD, effectuées soit avant soit après vaccination. Toutefois, une corrélation significative a été notée entre la réponse tardive à la lépromine et la réponse à l'épreuve cutanée au MLSA pratiquées après la vaccination. En général, aucune association n'a été trouvée entre les épreuves cutanées *in vivo* et l'épreuve de transformation lymphocytaire *in vitro* (LTT). Dès lors, on peut conclure que l'épreuve à la lépromine représente essentiellement une vaccination qui provoque une réponse spécifique aux antigènes de *M. leprae,* à condition que la dose de lépromine de tatou soit supérieure à 5×10^7. Cette épreuve ne convient donc pas comme épreuve diagnostique pour la lèpre. La variabilité des réponses précoces à la lépromine, particulièrement aux doses les plus basses, contre-indique son utilisation pour mesurer une sensibilisation préalable. Il n'en reste pas moins que la réponse tardive à l'épreuve à la lépromine, plus fidèle, peut encore servir à identifier les individus susceptibles.

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