CUTANEOUS DELAYED-TYPE HYPERSENSITIVITY RESPONSIVENESS IN LEPROMATOUS AND BORDERLINE LEPROMATOUS LEPROSY PATIENTS AS DETERMINED BY MULTITEST® CMI™*

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Abstract. To assess cell mediated immune (CMI) function in patients with lepromatous and borderline lepromatous leprosy (LL and BL), 35 patients were examined with the MULTITEST® CMI™ system to evaluate cutaneous delayed-type hypersensitivity (DTH) responsiveness to 7 recall antigens. Reactions were assessed quantitatively and qualitatively. In addition, patients were classified as "responsive"(≥ 2 positive reactions), "hypo-responsive" (1 positive reaction), or anergic. Only hyporesponsive and anergic patients were re-tested. In 23 patients tested before treatment started (Group 1), 9 were responsive, 4 hypo-responsive, and 10 anergic. Upon re-testing, 10 of the 14 hyporesponsive-anergic subjects showed improvement. In 12 patients assessed after therapy initiation (Group 2), 9 were responsive and 3 others became responsive upon re-testing. Quantitative assessment indicated variable deficiencies in cutaneous DTH reactivity that, in many cases, improved with therapy. Correlations between reactivity and disease severity (LL versus BL) or duration of disease were not observed.

The MULTITEST® CMI™ system provided a convenient, safe, and reproducible method to assess cutaneous DTH responsiveness in LL and BL patients. Our findings indicated that most LL and BL patients are able to generate detectable but generally fewer and less robust cutaneous DTH responses to recall antigens, many improving with therapy. However, a semi-quantitative classification whereby patients that reacted to 2 or more antigens were considered "responsive" showed little difference between patients and controls. Overall, the data support the contention that deficits in cutaneous DTH responsiveness probably neither predispose nor necessarily accompany lepromatous disease, a practical consideration as efforts to develop a leprosy vaccine continue.

INTRODUCTION

The lepromatous forms of leprosy, to include lepromatous leprosy (LL) and borderline lepromatous leprosy (BL), are associated with deficiencies in cell-mediated immunity (CMI) (anergy) against *M. leprae* as manifested by robust replication of *M. leprae* within permissive macrophages, reduced peripheral blood mononuclear cell proliferation upon exposure to *M. leprae*, and deficient lepromin (Mitsuda) skin tests (Sehgal, 1994). However, previous assessments of cutaneous delayed type-hypersensitivity (DTH) responsiveness in LL and BL patients when tested with non-leprosy, commonly used recall antigens have shown inconsistencies (Bullock, 1968; Guinto, 1968; Rea *et al*, 1976; Ulrich *et al*, 1972). This may relate to differences in the type or concentration of antigens used, methods of application, interpretation, or analysis. In addition, lepromatous disease may be associated with variable responsiveness among individuals, depending on bacterial load, duration of disease before therapy was started or genetic factors such as ethnicity or histocompatibility classification (Rea *et al*, 1976).

The MULTITEST® CMI™ is a disposable, plastic applicator with 8 heads containing tines pre-loaded with 7 common recall antigens and a control substance (vehicle) that provides an accurate, reproducible, convenient, and safe method to assess cutaneous DTH responsiveness (Ahmed and Blose, 1983; Frazer *et al*, 1985; Walsh *et al*, 1995). The localized reactions generated by standardized anti-

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gen preparations administered percutaneously by tines are readily quantified and as importantly, may reduce some of the potential pitfalls in skin testing by providing consistent antigen doses and depth of application. Here, we used the MULTITEST® CMI™ to assess the capability of LL and BL patients, before or after therapy initiation, to generate cutaneous DTH responses to recall antigens. Our data indicate that LL and BL patients are variably deficient but not necessarily incapable of generating DTH reactions to common recall antigens, and that therapy generally improves responsiveness. Thus, we concur with the contention that defects in cutaneous DTH reactivity probably neither accompany nor necessarily predispose to the development of leprosy (Rea *et al*, 1976), a concept that may be relevant as efforts to develop a leprosy vaccine continue (Nath, 1998).

PATIENTS AND METHODS

Patients

Filipino patients aged 14 to 60 years old and diagnosed with LL or BL (multibacillary) at the Leonard Wood Memorial Center for Leprosy Research were eligible for enrollment. Reasons for exclusion included pregnancy, other known systemic diseases, reactional leprosy (erythema nodosum leprosum or reversal reaction), or immuno-suppressive therapy within the previous 6 months. The study was approved by the Institutional Review Board of the Leonard Wood Memorial.

Patients were classified according to the Ridley-Jopling scale (Ridley and Jopling, 1966). Lepromatous patients were subcategorized into lepromatous (LL) or borderline lepromatous (BL) based on clinical examinations, slit skin smears from 6 sites stained for acid fast bacilli (bacterial index, BI) and histological findings. Clinical classifications took precedence over histopathology when inconsistencies occurred. Controls consisted of volunteers from patient communities similar in age and sex to the study group, without clinical signs of leprosy. Two patients with untreated borderline tuberculoid (BT) leprosy were also tested.

Cutaneous delayed type hypersensitivity testing

Skin testing was performed upon diagnosis prior to starting therapy (Group1) or after therapy had started (Group 2) using the MULTITEST® CMI™ (Pasteur Merieux, Lyon, France) (Fig 1). The MULTITEST® CMI™ consists of a disposable plastic applicator with 8 sterile test heads (9 tines per head) pre-loaded with 7 recall antigens (standardized in sensitized guinea pigs) and a negative control: tetanus toxoid, diphtheria toxoid, *Streptococcus pyogenes* (Group C), old tuberculin, *Candida albicans*, *Trichophyton mentagrophytes*, *Proteus mirabilis*, and glycerin (the vehicle for the test antigens). The skin test was applied to the alcohol disinfected anterior surface of the forearm with firm pressure for 30 seconds according to the manufacturer's instructions. Following application, the test substances were allowed to remain on the skin surface for at least 3 minutes and then gently dabbed with a gauze pad in a manner to avoid cross contamination. The test sites were then read and scored at 48 hours by one experienced reader (LGV).

Reactions were graded as positive only if the induration at the inoculation site was at least 2 x 2 mm in size (Fig 2). Erythema without induration was ignored. Results were recorded as both the number of antigens positive as well as the cumulative mm (length + width (2) of reaction for all 7 antigens. In addition, a semi-quantitative method was used to classify patients as "responsive" if ≥ 2 antigens were positive, "hyporesponsive" if 1 antigen was positive, or "anergic" if all antigens were negative. "Responsive" (≥ 2 antigens positive) patients were not routinely re-tested, whereas hyporesponsive and anergic patients were re-tested at least once, at not less than 3 month intervals. Any hyporesponsive or anergic patient not available for at least 1 re-test was considered "non-evaluable". Controls were tested only once.

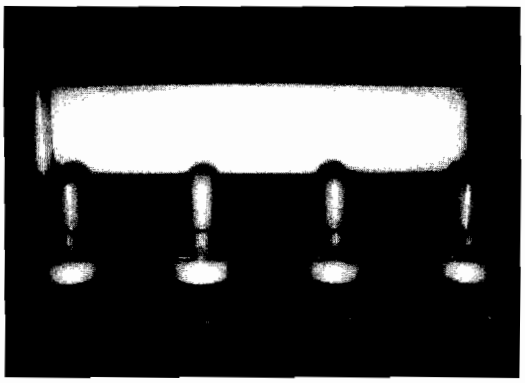

Fig 1–The MULTITEST® CMI™ system consists of 8 arms containing heads (tines) pre-loaded with 7 test antigens and a control substance. Before application, the clear plastic caps are gently removed to expose the tines.

Fig 2–Forty-eight hours after application of the MULTITEST® CMI™ to the anterior forearm, 4 test sites have developed induration of at least 2 x 2 mm (arrows) and are graded as positive reactions.

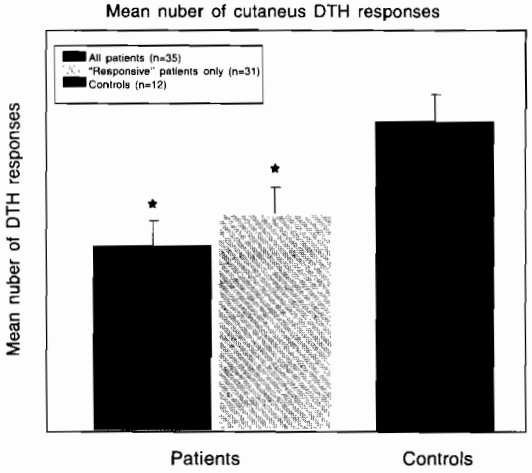

Fig 3–Comparison of the mean (± SEM) number of cutaneous DTH responses in all patients (black bar), responsive patients only (light gray bar), and controls (dark gray bar).

* represents a significant difference from control values ($p < 0.05$, Mann-Whitney U test).

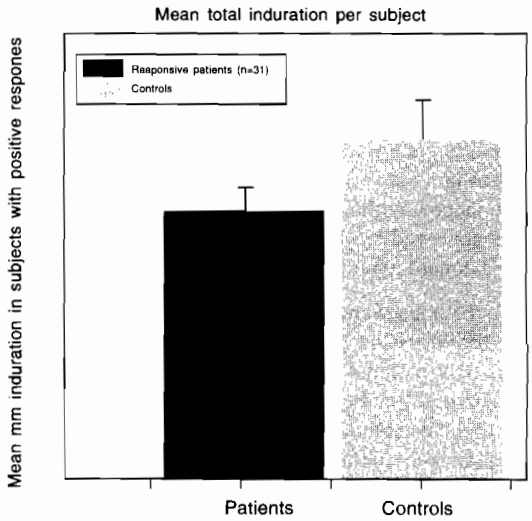

Fig 4–Comparison of the mean mm (± SEM) of induration per subject (patients versus controls) for all cutaneous DTH reactions. The patient and control values were not significantly different.

Statistical analysis

Patients and contacts were compared by the non-parametric Mann-Whitney U test for independent samples. A p value of 0.05 or less was considered significant.

RESULTS

Patients

Thirty-five patients with leprosy, aged 14-54, were evaluated. Of these, 23 were newly diagnosed and tested before treatment initiation (Table 1). Twelve patients were first tested after treatment had started (Table 2).

Cutaneous delayed type hypersensitivity responsiveness

Quantitative analysis: Control subjects consisted of 5 males and 7 females with an age range of 26 to 63. The cutaneous DTH responses were similar between males and females. The mean number of positive responses was 3.7 and the mean mm induration per subject was 18.8 mm (Figs 3 and 4). Within the control group, the proportion of subjects responding to each antigen consisted of the following: *Candida albicans* (83%), tuberculin (83%), *Proteus mirabilis* (75%), *Diptheria toxoid* (67%), tetanus toxoid (58%), *Streptococcus pyogenes* (8%), and *Trichophyton mentagrophytes* (8%). There were no positive reactions to the glycerin control and no adverse reactions to skin testing were observed. The 2 untreated BT patients responded to 2 antigens each.

BL and LL patient responses are shown in Figs 3 and 4. Fig 3 shows that the mean number of positive

Table 1
MULTITEST® CMI™ results in leprosy patients first tested **before** therapy initiated.

	Pt	Age	Sex	Dis Dur	Clin Dx	Histo Dx	BI	Tx	No. of antigens positive		
									1st test	Repeat testing (months of therapy)	
First test:											
Responsive											
	EA	32	F	3 y	LL	LLs	4.0	MDT	2	ND	
	RB	24	M	5 y	LL	LLs	4.7	MDT	5	ND	
	JC	24	M	7 y	BL	BL	3.7	MDT	2	5 (24 m) ("random" re-test)	
	PD	32	M	1 y	LL	LLs	4.3	MDT	2	ND	
	RG	24	M	2 y	LL	ND	5.0	MDT	4	ND	
	RL	35	M	5 y	BL	BB	3.8	MDT	2	5 (20 m) ("random" re-test)	
	NM	21	F	2 y	LL	LLs	2.8	MDT	4	ND	
	JQ	19	F	1 y	LL	LLs	5.0	ROM	2	ND	
	VR	25	M	5 y	LL	ND	3.0	MDT	3	ND	
Hypo-responsive											**Improved reactivity (3 of 4)**
	PD	28	M	5 m	BL	BB	4.3	MDT	1	4 (22 m)	
	DD	28	M	5 y	LL	LLs	4.3	MDT	1	0 (12 m)	2 (15 m)
	EO	22	M	1 y	BL	BL	4.0	ROM	1	4 (6 m)	
	ER	20	M	1 y	LL	LLs	3.8	MDT	1	1 (13 m)	1 (16 m)
Anergic											**Improved reactivity (6 of 10)**
	PF	22	M	1 y	BL	ND	4.7	MDT	0	2 (6 m)	2 (13 m)
	JM	31	M	2 y	LL	LLs	5.0	MDT	0	4 (6 m)	
	SJ	36	M	4 y	LL	LLs	5.0	MDT	0	0 (3 m)	0 (6 m) 3 (25 m; Tx completed)
	CS	14	M	2 y	LL	LLs	4.6	MDT	0	0 (3 m)	2 (23 m)
	ET	27	M	7 y	LL	LLs	4.2	MDT	0	5 (18 m)	
	PG	22	F	2 y	BL	ND	4.7	MDT	0	1 (15 m)	0 (24 m; Tx completed)
											Remained anergic (4 of 10)
	EF	21	M	1 y	BL	BL	2.2	MDT	0	0 (12 m)	
	RI	31	F	5 y	BL	BL	5.0	MDT	0	0 (21 m)	0 (24 m; Tx completed) 0 (30 m)
	VR	19	F	3 y	LL	ND	4.2	MDT	0	0 (2 m)	0 (13 m)
	JS	16	M	2 y	BL	ND	2.2	MDT	0	0 (14 m)	0 (17 m)

Table 2
MULTITEST® CMI™ results in leprosy patients first tested after therapy initiated.

	Pt	Age	Sex	Dis Dur	Clin Dx	Histo Dx	BI at Dx	Tx	Months of Tx at 1st test	No. of antigens positive	
										1st Test	Repeat Testing (time)
First test:											
Responsive											
	BB	43	M	3 m	BL	BT	2.7	MDT	12 m	2	ND
	AB	25	M	5 m	BL	LLs	4.7	MDT	2 m	5	ND
	JE	23	M	8 y	LL	LLs	3.7	MDT	2 m	2	ND
	Al	35	M	3 y	LL	LLs	4.8	MDT	21 m	2	3 ("random" re-test, 6 m after Tx completed)
	EM	27	M	1 y	LL	LLs	4.6	ROM	9 m	3	ND
	DP	25	M	2 y	LL	LLs	4.3	MDT	4 m	2	ND
	AP	22	M	1 y	LL	LLs	4.5	MDT	12 m	3	ND
	MT	27	M	5 m	LL	LLs	4.8	MDT	3 m	3	ND
	RT	25	M	2 y	LL	LLs	3.3	MDT	11 m	7	ND
Hypo-responsive											**Improved reactivity (3 of 3)**
	FM	34	M	2 y	LL	LLs	4.0	MDT	3 m	1	2 (Tx dur: 17 m)
	CS	46	M	10 y	LL	LLs	5.3	ROM	4 m	1	2 (4 m after Tx completed)
Anergic											
	AN	24	M	3 y	LL	LLs	4.5	MDT	3 m	0	0 (Tx dur: 16 m) 5 (Tx dur: 20 m)

Abbreviations used in Tables 1 and 2: Pt (patient), Dis (disease), Dur (duration), Clin (Clinical), Dx (diagnosis), Histo (histological), BI (average bacterial index), Tx (treatment), MDT (WHO-Multidrug Therapy), ROM (rifarnpicin-ofloxacin-minocycline), LL (lepromatous leprosy), LLs (subpolar lepromatous leprosy), BB (borderline leprosy), BT (borderline tuberculoid leprosy), ND (not done)

responses among patients were significantly reduced in comparison with controls (p = 0.03) and Fig 4 shows the comparison of the mean induration of positive reactions between patients and controls (p = 0.09).

Semi-quantitative analysis :

Patients first tested before therapy started (Group 1)

The results of cutaneous DTH testing in Group 1 are summarized in Table 1 and Fig 5. Upon completion of all testing in 23 patients, 18 were responsive, 1 was hypo-responsive, and 4 remained anergic. Average BIs among the different response categories were not significantly different. Most patients were treated with WHO-Multidrug Therapy (MDT) consisting of dapsone, rifampicin, and clofazimine, whereas others received a regimen of rifampicin, ofloxacin, and minocycline (ROM) (Gelber *et al*, 1995; Walsh *et al*, 1989). Two patients that reacted to 2 antigens on initial testing were randomly selected for re-testing, and each showed 5 positive reactions. Patient "PG", anergic on initial testing, demonstrated 1 positive reaction after 15 months of therapy but then no positive reactions at 24 months upon treatment completion. Within Group 1, the proportion of subjects responding to each antigen consisted of the following: tuberculin (79%), *Candida albicans* (58%), *Diptheria toxoid* (47%), *Streptococcus pyogenes* (39%), tetanus toxoid (26%), *Proteus mirabilis* (26%), and *Trichophyton mentagrophytes* (21%). There were no positive reactions to the glycerin control and no adverse effects of skin testing observed. There was no correlation between disease classification (LL versus BL) or duration of disease prior to treatment and responsiveness.

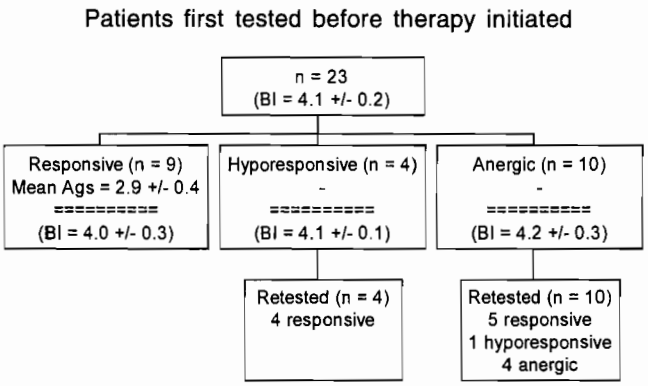

Fig 5–Flow chart depicting the mean number of cutaneous DTH responses and mean bacterial indexes (BI) in 23 **untreated** patients and follow-up testing with MULTITEST® CMI™. Mean BIs among responsive, hyporesponsive, and anergic patients were not significantly different.

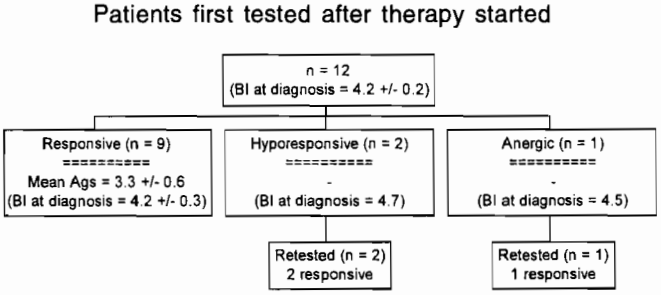

Fig 6–Flow chart depicting the mean number of cutaneous DTH responses and mean bacterial indexes (BI) in 12 patients tested with MULTITEST® CMI™ **after** therapy had been initiated.

Patients first tested after therapy initiation (Group 2)

The results of cutaneous DTH testing in Group 2 are summarized in Table 2 and Fig 6. Of 12 evaluable patients, 9 were responsive on first testing, and 3 became responsive upon re-testing. On first testing, average BIs of the hyporesponsive and anergic patients (n = 3) were slightly higher than the responsive patients. Most patients were being treated with WHO-MDT; several others had been placed on an ROM regimen. Within Group 2, the proportion of subjects responding to each antigen consisted of the following: tuberculin (66%), *Candida albicans* (58%), *Streptococcus pyogenes* (42%), tetanus toxoid (42%), *Proteus mirabilis* (42%), *Trichophyton mentagrophytes* (42%), and diptheria toxoid (37%). There were no positive reactions to the glycerin control and no adverse effects of skin testing observed. Correlations between disease classification (LL versus BL) or duration of disease prior to treatment and responsiveness were not observed.

The semi-quantitative analysis is summarized in Fig 7. Eleven of 12 controls (92%) were "responsive" and 1 of 12 (8%) was "hyporesponsive". In the leprosy patients, 30 were "responsive" (86%), 1 "hyporesponsive" (3%), and 4 remained anergic (11%). The percentage of "responsive" controls versus leprosy patients was not significantly different (p = 0.9).

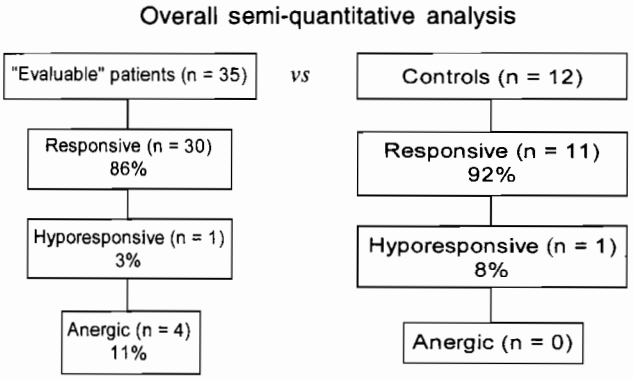

Fig 7–Overall comparison of LL and BL patients with controls by a semi-quantitative grading scale of responsive, hyporesponsive, or anergic. The percentage of responsive controls versus patients was not significantly different.

DISCUSSION

In tuberculoid leprosy patients, CMI responsiveness to *M. leprae* as well as to non-leprosy antigens is generally strong (Guinto, 1968). In contrast, LL and BL patients are characterized by abnormal CMI responses to *M. leprae*, and in some studies, to non-leprosy antigens as well, suggesting generalized CMI deficiencies (Rea *et al*, 1976). Indeed, LL and BL patients, especially before treatment, demonstrate variable responsiveness when skin tested with recall antigens (Bullock, 1968; Convit *et al*, 1971; Guinto, 1968; Rea *et al*, 1976; Ulrich *et al*, 1972). Here, we used a commercially available plastic applicator composed of tines pre-loaded with 7 recall antigens to improve standardization of DTH testing in leprosy patients. The responses, assessed in quantitative and semi-quantitative fashion, confirm that LL and BL patients are variably deficient but not necessarily incapable of generating cutaneous DTH responsiveness that, in some cases, improved with therapy. That some patients demonstrated a degree of responsiveness before therapy, that others responded after therapy initiation or completion, and overall, that 86% of the patients were found to respond to 2 or more antigens, supports the contention that altered cutaneous DTH responsiveness probably neither accompanies nor predisposes to the development of leprosy, but represents remote sequelae (Rea *et al*, 1976).

Cutaneous DTH testing to recall antigens is a well established, *in vivo*, functional assay to evaluate CMI responsiveness (Ahmed and Blose, 1983). Typically, antigens are applied by intradermal inoculation (Mantoux technique) and injection sites are read 24 to 48 hours later. Although most cutaneous DTH investigations, including those in leprosy, have generally used intra-dermal inoculation of a large dose of two to four antigens, this technique is subject to inconsistencies such as variation in antigen dose and depth of inoculation (Ahmed and Blose, 1983; Frazer *et al*, 1985; Knicker *et al*, 1979). The MULTITEST® CMI™ administers 7 antigens with tines that traverse the epidermis and upper dermis (Frazer *et al*, 1985; Knicker *et al*, 1979). A major advantage of the MULTITEST® CMI™ involves the simultaneous application of multiple antigens, overcoming problems of individual variation affecting responsiveness to a single antigen challenge (Frazer *et al*, 1985; Knicker *et al*, 1979). In addition, consistent intracutaneous doses of specific antigen affords a standardized degree of exposure to Langerhans and dermal dendritic cells, increasing the probability of an accurate, reproducible DTH reaction. Thus, the number of antigens, the dose of antigen, the mode of application, and individual variability all combine to influence the clinical outcome of cutaneous DTH testing. Here, MULTITEST® CMI™ provided a reliable, convenient, and safe method to assess DTH in leprosy patients.

Abnormal cutaneous DTH responses may be found in post-surgical and trauma patients, malnutrition, HIV, and many other chronic diseases (Ahmed and Blose, 1983). Generally, declining health and loss of immune system responsiveness leads to a progressive decline of the DTH response and may herald imminent infection. Indeed, cutaneous DTH testing has been used in staging HIV positive patients and had been found useful in assessing disease progression (Redfield *et al*, 1986). In contrast to chronic diseases, however, LL and BL patients displayed variable patterns of responsiveness that frequently improved with therapy. Nonetheless, that four subjects remained anergic, but otherwise remained healthy and responded well to therapy suggests that leprosy may permanently alter cutaneous DTH capability or that higher antigen concentrations may be required in some patients to generate detectable responses. Alternatively, further re-testing at later times may have demonstrated improved DTH responsiveness.

As an adjunct to the quantitative MULTITEST® CMI™ scoring system, we also adopted a semi-

quantitative grading system whereby patients reacting to 2 or more antigens were classified as "responsive". Based on previous findings that treatment may improve CMI function in leprosy patients (Bullock, 1968), we hypothesized that untreated patients severely deficient or anergic for DTH reactivity might improve with treatment. Thus, we aimed to economize MULTITEST® CMI™ kits to the extent of focusing on "hyporesponsive" (1 positive reaction) or anergic subjects, as per this modified grading system. Moreover, although 2 positive responses are less than the mean of most normal control groups (approximately 3.5 positive responses) (Frazer *et al*, 1985; Knicker *et al*, 1979; Walsh *et al*, 1995), we propose that the capability to generate detectable DTH responses to 2 or more distinct antigens indicates relatively intact, functional CMI responsiveness that should not necessarily be considered "deficient".

Previous descriptions of cutaneous DTH responsiveness in patients with lepromatous disease have shown highly variable response patterns (Bullock, 1968; Convit *et al*, 1971; Guinto, 1968; Rea *et al*, 1976; Ulrich *et al*, 1972). These discrepancies may have been in part due to differences in the test antigens, doses, methods of application or interpretation, ethnicity, the duration of disease prior to diagnosis, the duration of therapy at testing, the presence of other concurrent infections (malaria, tuberculosis, etc), immunomodulatory therapy for reactional states, or other factors such as histocompatibility classifications (Bullock, 1968; Convit *et al*, 1971; Guinto, 1968; Rea *et al*, 1976; Ulrich *et al*, 1972). Here, using the MULTITEST® CMI™ system in a well characterized group of Filipino leprosy patients, we found variable degrees of reactivity, especially in the untreated patients, that in many cases improved with therapy. However, when assessed by a semi-quantitative grading system, deficiencies were relatively mild.

A cutaneous DTH response requires sensitization, followed by elicitation on re-exposure to the antigen. Elicitation is comprised of several complex events, any of which could be affected by the substantial *M. leprae* burden in LL and BL patients. Sensitized T cells must recognize antigen expressed on the surface of Langerhans cells and secrete lymphokines to initiate blastogenesis, further release of other cytokines, and recruitment of more lymphocytes and macrophages. Paramount to this cascade is recruitment of CD4+ memory T cells, secretion of interleukin-2 (IL-2), and expression of IL-2 receptors (Villahermosa *et al*, 1997). Each of these events may be adversely affected in lepromatous disease and may involve: i) a direct effect of *M. leprae* on T or Langerhans cells, ii) decreased numbers of Langerhans cells or dermal "surveillance" T cells, or iii) an abundance of circulating leprosy antigens, such as PGL-1, overwhelming the capability to mount a detectable CMI response ("immune distraction") in a polarized, Th2-like predominant milieu (Cho *et al*, 1991; Kaplan and Cohn, 1991). Thus, it would not be unexpected that killing of *M. leprae* by chemotherapy might restore or gradually improve cutaneous DTH responses as observed here.

The practical implications of these observations may be relevant as efforts to develop a leprosy vaccine continue (Nath, 1998). Because a successful vaccine must generate strong CMI responses against *M. leprae*, persons who may otherwise develop lepromatous disease after exposure to the organism must be capable of generating protective Th1-like immune responses upon vaccination. Our data suggest that given the proper antigen or combination of antigens, potentially susceptible persons should be able to generate strong CMI responses that hopefully will lead to protection against leprosy.

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