

Field Evaluation of an ELISA to Detect Antibody in Leprosy Patients and Their Contacts¹

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Humoral antibodies have been detected in leprosy patients, particularly in multi-bacillary disease, and in some of their contacts by an increasing number of laboratories using a variety of antigens and a variety of methods, including radioimmunologic assays, immunodiffusion gel plates, crossed immunoelectrophoresis, fluorescent tagging, and enzyme-linked immunosorbent assays (ELISA) (1-6, 8-10).

Since no proven *Mycobacterium leprae* "specific" antigen has been available until recently (3, 11), humoral antibody studies have been limited to antigens which contain some "common" components shared by other mycobacteria. Attempts have been made to block crossreacting antigens to improve specificity. Abe's FLA-ABS method has been used in field surveys and indicates a rather wide prevalence of antibody in non-leprosy cases in populations with a low prevalence of disease (?). There is no report of serial studies of individual people in exposed populations to estimate the "predictive value" of sero-conversion.

In his recent review of leprosy antibody studies, Harboe (7) states:

. . . if we can provide antibody assays for reliable identification of single individuals at this [preclinical] stage . . . and if they can be effectively treated by intensive combined chemotherapy . . . we may have a new and . . . efficient way of preventing the spread of the disease.

The primary purpose of the study reported here has been to follow such people in order to establish serologic and clinical correlations over time. Very high prevalence populations with limited geographic mobility have been described in Ponape, Micronesia (12-14). This preliminary report

describes the beginning of a prospective study of that population, using an ELISA system to identify incipient new cases, especially multibacillary cases.

METHODS

Laboratory

The early stages of ELISA development which led to the selection of autoclaved *M. smegmatis* (TMC strain #1515) whole bacilli as the initial antigen for field testing (supplies of *M. leprae* were not then adequate) was done with sera from well-classified leprosy cases in Hawaii (5).

In Hawaii, sera are usually obtained from venipuncture blood and then stored frozen until diluted for processing. On Ponape, blood from finger-prick is dispensed from a heparinized microhematocrit capillary tube in 25 μ l aliquots onto each of three filter paper disks (S&S #740-E $\frac{3}{8}$ inch diameter antibiotic sensitivity filter paper disks), allowed to dry overnight, then stored frozen until each disk is thawed in 0.5 ml half-normal saline with 0.1% sodium azide to produce eluate (approximately 1:20 plasma dilution) for further processing in the ELISA system.

Antigens are suspended in standard optical density concentrations, coated onto wells in flat-bottom microtiter plates, and then dried. Diluted sera or eluates are then added to duplicate wells, incubated, and then washed four times before adding horseradish peroxidase enzyme conjugated to anti-human IgGAM. After 30 min of incubation, the reaction is stopped by adding sulfuric acid and then read in a Titertek Multiskan (Flow Laboratories, Inc., Rockville, Maryland, U.S.A.) at OD₄₉₂ nm.

Reactivity is expressed as the optical density (OD) of pooled negative sera (or mean of negative eluates) subtracted from the OD of test sera. Each plate contains pooled-positive and known-negative wells, and an algorithm is used to divide the region between the OD for the negative and positive con-

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trols into five zones, "negative" or 1+ to 4+. [The algorithm is as follows: $D = (OD \text{ pos. control}) - (OD \text{ neg. control})$. Neg. upper limit (NUL) = $OD \text{ neg. control} + 0.08D$; $UL \ 1+ = NUL + 0.2D$; $UL \ 2+ = NUL + 0.4D$; $UL \ 3+ = NUL + 0.6D$.] Test sera are delivered into two wells, and the average OD for the paired wells is reported unless the pair differ from each other by 10%, in which case that specimen is ignored and is rerun on another plate.

Field

The Department of Health (DOH) of Ponape agreed to collaborate in this study, and a graduate student from Hawaii was sent to Ponape in the summer of 1980 to assist their DOH in a preliminary round of blood collection and leprosy screening. That survey covered 266 people and identified a new, rapidly increasing epidemic in a previously leprosy-free subpopulation (¹⁷).

Among these 266 people, all of whom lived on the island of Ponape, there were 257 who had neither current signs of nor a history of leprosy. By the summer of 1982 sufficient funds were available to send a team of three graduate students back to Ponape to help the DOH do a wider screening of leprosy, covering both Ponape and the atoll of Kapingamarangi, the village of origin of the subpopulation with the new epidemic. Pingelap atoll was not covered due to transportation problems.

During these surveys, blood specimens and examinations were obtained for 1573 nonleprosy cases (non-cases) and 175 cases or suspects. Skin biopsies were obtained from 86 cases and suspects and were sent to the National Hansen's Disease Center (NHDC), Carville, Louisiana, U.S.A., for examination. An additional 78 biopsies were obtained during the fall and winter of 1982, with a re-survey of about 900 people. The histopathologic classification given by a highly experienced pathologist at the NHDC was the basis for case classification in this study. Clinical screening was done by the same leprosy nurse who worked annually with two highly experienced leprologists used in the 1967-1977 studies on Ponape. The same pathology confirmation rate (about 90-95%) found in those studies is being obtained in the current series, with disagreement almost exclusively confined

to failure of biopsy confirmation of early indeterminate (I) or tuberculoid (TT) lesions seen clinically. These are carried by us as "suspects."

During the summer of 1982, 228 people who had been among the 257 non-cases seen in the summer of 1980 were identified. The two-year follow up of these 228 people is the main focus of this report.

RESULTS

The finger-prick, three filter-paper disk, blood collection method was acceptable to subjects and easy to teach to field workers. The disks were easy to store and ship. Their use, however, adds one additional step to the processing procedures in the laboratory.

Table 1 displays a cross-tabulation of paired ELISA results from 104 sera collected in 1980 and tested after seven months and 23 months of freezing as blood-soaked filter-paper disks. There is very little evidence (one pair out of 104) of loss of titer with freezing, but there is some evidence (nine pairs) of increases of titer, probably relating to improving ELISA sensitivity as the technique evolved during 1981 and 1982.

There is evidence (J. T. Douglas, unpublished data) that the *M. smegmatis* antigen also reacts with sera from some active tuberculosis cases (especially cavitary). Therefore 47 people on Ponape who were found to be ELISA positive in the summer of 1982 were given tuberculin skin tests ("Tine," Lederle Laboratories, Pearl River, New York, U.S.A.) in December of 1982. Only four of these were tuberculin positive (all in the 10 mm-12 mm equivalent level), and none had signs of active tuberculosis. It seems unlikely, therefore, that the data presented below are significantly distorted by crossreactions due to *M. tuberculosis* infections, nor to the use of BCG (not used on Ponape since 1962).

Prevalence data

Table 2 distributes the ELISA reactions found in 1982 in 150 biopsy-proven cases of leprosy by histopathological type and by year of onset. Although the numbers of cases are small in some categories, there is a suggestion of: a) high initial ELISA level in all multibacillary cases, followed by a slow fall in most; b) lower initial levels in most pau-

TABLE 1. *ELISA results with M. smegmatis antigen for 104 paired eluates from blood-soaked filter-paper disks frozen for seven and 23 months after collection from people exposed to leprosy in Ponape, Micronesia, 1980.*

		ELISA results after 23 months of freezing				
		Negative	1+	2+	3 - 4+	Totals
ELISA results after 7 months of freezing	3 - 4+	0	1	0	5	6
	2+	0	4	4	5	13
	1+	2	5	1	2	10
	Negative	51	17	5	2	75
Totals		53	27	10	14	104

①	pair showing significant loss of titer
⑨₄	pairs showing little change
⑨	pairs showing significant increase in titer

cibacillary cases, followed by a probably more rapid fall; and c) an upward drift (or persisting high levels) in a few cases, possibly indicating reactivation or poor response to treatment.

Table 3 distributes 1573 non-cases by subpopulation and by ELISA level in the summer of 1982. Each of the "epidemic" groups shows a prevalence rate of 43% ELISA positive; while in contrast another population on Nukuor, a nonendemic atoll visited by Kapinga cases, showed only 3 positives out of 80 people (4%), all 3 having

had case contact. The low Nukuor positive rate is an indication that crossreaction due to tuberculosis or environmental mycobacteria is probably not an important factor in the 43% positive level found in the "epidemic" groups.

Incidence data

The real test of usefulness of a screening test lies in its ability to predict disease. Table 4 distributes 228 non-cases seen in the summer of 1980 and again two years later. The 70 people who had initially been ELISA

TABLE 2. Distribution of 150 biopsy-proven cases by type of leprosy, year of onset, and serologic status, summer 1982, in Ponape, Micronesia.

ELISA level	Year of onset						Totals
	1982	1981	1980	1979	1978	<1978	
Multibacillary							
3-4+	1	6				5	12
2+	2				1	3	6
1+		1 ^a	1	1		4	7
Negative			1 ^a			4	5
Total							30
Paucibacillary							
3-4+	3				2	5	10
2+	10	2		1		2	15
1+	20	9	1			9	39
Negative	32	13	1			10	56
Total							120

^a Borderline case.

positive but whose ELISA level had fallen by 1982 (the "healers") suffered a 9% average annual attack rate, all paucibacillary, including four borderline tuberculoid (BT) cases. The 68 who were initially positive and whose levels persisted or rose in 1982 ("sero-persisters") suffered a 10% average annual attack rate, including a new lepromatous (LL) case. The 59 who were seronegative at both screenings ("negatives") suffered a 4% average annual attack rate, all I or TT. The 31 who were negative in 1980 but who were positive in 1982 ("converters") suffered a 10% average annual attack rate, including 1 BL and 1 LL case.

If we hypothesize that three of the five "negative" cases would have been found as ELISA 1+ or 2+ temporarily if screened in the summer of 1981, then the average annual attack rate among the "negatives" would fall to 2%, and that for the "converters" would rise to 13%. This is a 6 to 1 "relative risk" among the "converters."

Table 5 distributes the same data as that in Table 4 but shows a right marginal estimate of the average annual attack rates for each level of ELISA reactivity found in 1980, without regard for 1982 results. If the same adjustment is made here for the "negatives," the average annual attack rates for all those found sero-positive with an hypothesized annual screening interval would

TABLE 3. Distribution of 1573 people without a history or signs of leprosy, by ELISA results and by subpopulations, Ponape, Micronesia, summer 1982.

ELISA level	Pingelapese	Kapinga	Nukuor
4+	12 (1.6%)	4 (0.5%)	0
3+	25 (3.4%)	6 (0.8%)	0
2+	73 (9.9%)	64 (8.5%)	0
1+	210 (28%)	249 (33%)	3 (4%)
Negative	418 (57%)	432 (57%)	77 (96%)
Totals	738	755	80

be about 10%. This is the "predictive power positive" and is dependent, in part, on the incidence of the disease in the population being screened.

DISCUSSION

These preliminary data indicate that an ELISA screen in this high-incidence population can identify 70% of impending (pre-clinical) cases at a two-year screening interval, and over 90% (including all multibacillary cases) at a one-year interval. (See Table 5. With the one-year adjustment, 35 out of 37 new cases would probably be found sero-positive prior to clinical signs.) *M. smegmatis*, which contains bacterial antigens "common" to *M. leprae*, will soon be replaced by an antigen "specific" for *M. leprae*, either in combination with "common" antigens (such as whole *M. leprae* bacilli) or alone (such as glycolipid antigen now becoming available) (3, 11).

If a specific antigen alone becomes feasible, then it can be substituted directly for the *M. smegmatis* in the two-well system currently used. If, however, only a combined antigen is feasible, then a two-well screening system can be continued with such an antigen but a third well will need to be added, in which the specific antigen is blocked by specific antibody prior to adding the test sera, in order to identify "false positives" found in the screening pair of wells.

In either case, we will then rerun the improved ELISA on all of the critical sera from our 1980 survey, the 1982 summer and winter surveys, and the two additional six-month-interval surveys planned for the summer and winter of 1983.

It is our current impression that future

TABLE 4. Paired ELISA results and leprosy attack rates among 228 non-cases examined and bled in June 1980, and again two years later in Ponape, Micronesia.*

		Serologic patterns		
		ELISA results in June - July, 1982		
		Negative	1+	2 - 4+
ELISA results in June, 1980	2 - 4+	<u>Falling titers</u> 70 people ("healers") 12 new cases, all paucibacillary (9%)**		<u>Persistent or rising</u> ("positives") 68 people 14 new I, T, BT, LL cases (10%)**
	1+	<u>"Negatives"</u> 59 people 5 new I, T cases (4%/2%***		<u>Seroconverters</u> 31 people ("converters") 6 new I, BT, BL, LL cases (10%/13%***
	Negative			

* = Data subject to slight change when remaining biopsy reports received.

** = Average annual attack rates.

*** = Adjustments made on the shift of the 3 1980 seronegative cases who had the onset of a minor I or TT lesion in late 1981 to a probable 1+ ELISA result if rescreening had been done in Summer, 1981.

work will show that the most efficient use of this serologic tool in making highly accurate predictions of impending disease will employ a strategy that is a modification of that used for Table 4. All new "converters" found during a serially repeated survey in an exposed group should be re-screened within a few (3-6) months after the conversion is discovered in order to separate by titer pattern those who are "healers" from those who are "sero-persisters" who are at a higher risk of clinical disease and among whom will be all of the incipient multibacillary cases.

It is also likely that the perfected ELISA technique will be more easily "decentralized" to regional laboratories in endemic

areas than is likely for more technically difficult assay methods.

The average attack rate for the entire 1980 cohort of 228 people over the two-year period was 8% per year. This is far higher than the cumulative ten-year attack rate of about 10% observed in household contacts of lepromatous parents in Hong Kong and Hawaii^(15, 16), and may reflect "volunteer bias" in the 228 who stepped forward in 1980.

SUMMARY

Previous studies have detected circulating antibody in leprosy using a variety of difficult laboratory methods. We have developed a simpler method for detecting antibody by ELISA, using autoclaved *Mycobacterium*

TABLE 5. Paired ELISA results and leprosy attack rates among 228 non-cases examined and bled in June 1980, and again two years later in Ponape, Micronesia.*

		2-year cohorts (# new cases / 1980 serologic status)			(Average annual attack rates)
		ELISA results in June - July, 1982			
		Negative	1+	2 - 4+	
ELISA results	2 - 4+	2/13	(2BT) 4/21	(3BT, 1LL) 6/24	(10.3%)
	1+	6/36	7/32	(BL) 1/12	(8.8%)(10.2%)**
in June, 1980	Negative	5/59	(1BT) 3/22	(1BL, 1LL) 3/9	(6.1%)(4.4%)**
		↑**			→

* Data subject to slight change when remaining biopsy reports received.

** Adjustments made on the shift of the 3 1980 seronegative cases who had the onset of a minor I or TT lesion in late 1981 to a probably 1+ ELISA result if rescreening had been done in Summer, 1981.

bacterium smegmatis as the antigen. Evaluation was performed on eluates from 25 μ l aliquots of finger-prick blood dried on filter-paper disks in two high-incidence populations in Ponape, Micronesia. Among 228 nonleprosy cases bled in 1980 and re-bled and re-examined in 1982: a) for those who had been ELISA positive two years earlier, the leprosy attack rate during the intervening two years was at least twice as high as among those who had been negative, and we estimate that shortening the screening interval to one year plus doing confirmatory retests on new sero-converters would increase the relative risk (or "predictive power") to over sixfold, including all impending multibacillary cases; b) elevated antibody levels were detected up to two years prior to clinical onset of disease in 70% of new cases; and c) both asymptomatic conversion (rising titer) and reversion (falling titer) were observed.

Among 150 biopsy-proven cases, ELISA results suggest that fall of titer in most uncomplicated paucibacillary cases was rapid (months), but in multibacillary cases was more gradual (years), probably paralleling

responses to treatment with titers rising in reactivation.

These results suggest that this technique, with an improved antigen, may be useful in leprosy control programs, both for detecting candidates for preventive treatment and for following responses to therapy.

RESUMEN

En estudios previos se ha demostrado la presencia de anticuerpos circulantes en pacientes con lepra usando una variedad de métodos difíciles de laboratorio. Nosotros hemos desarrollado un método más simple para la demostración de anticuerpos por ELISA, usando como antígeno al *Mycobacterium smegmatis* sometido al autoclave. El estudio se hizo con alícuotas de 25 μ l tomadas de los eluidos de pequeños discos de papel filtro previamente impregnado con gotas de sangre de habitantes de 2 zonas de alta incidencia en lepra en Ponape, Micronesia. Entre los 228 individuos sangrados en 1980 y vueltos a sangrar y a examinar en 1982: a) para aquellos casos que fueron positivos por ELISA dos años antes, la incidencia de lepra durante los siguientes 2 años fue cuando menos 2 veces más alta que en aquellos que habían sido negativos, y nosotros calculamos que acortando al intervalo de búsqueda a un año, además de hacer pruebas confirmatorias en los nuevos casos sero-convertidos, el

incremento en el riesgo relativo (o "poder predictivo") podría ser mayor a 6 veces, incluyendo todos los casos multibacilares; b) se detectaron niveles elevados de anticuerpo hasta 2 años antes de la aparición clínica de la enfermedad en el 70% de los casos nuevos; c) se observaron tanto conversiones asintomáticas (incrementos en los títulos) como reversiones (caída en los títulos).

En un estudio de 150 casos comprobados por biopsia, los resultados por ELISA demostraron que la caída en el título en la mayoría de los casos paucibacilares no complicados fue rápida (meses), mientras que en los casos multibacilares la caída fue más gradual (años); esto probablemente refleje el efecto del tratamiento, con incremento en los títulos durante los estados reaccionales.

Estos resultados sugieren que esta técnica, con un antígeno mejorado, puede ser útil en los programas de control de la lepra, tanto para identificar a los candidatos de tratamiento preventivo, como para evaluar la respuesta al tratamiento.

RÉSUMÉ

Au cours d'études antérieures, on a pu détecter des anticorps circulant dans la lèpre au moyen d'une variété de méthodes de laboratoire laborieuses. On a développé dès lors une méthode plus simple pour déceler les anticorps par ELISA, en utilisant l'antigène *Mycobacterium smegmatis* autoclavé comme antigène. Dans deux populations de Ponape, en Micronésie, qui présentent une incidence élevée de lèpre, on a procédé à une évaluation de cette méthode, sur des éluats de 25 microlitres de sang récolté par ponction du doigt, et séché sur des disques de papier filtre. Parmi 228 témoins chez lesquels on avait recueilli des échantillons de sang en 1980, et de nouveau, après ré-examen, en 1982, on a fait les observations suivantes: a) parmi les malades qui étaient positifs pour ELISA deux ans auparavant, le taux d'attaque de la lèpre au cours des deux années suivantes a été au moins deux fois plus élevé que parmi les malades qui étaient négatifs; on estime qu'en réduisant à un an l'intervalle de temps entre deux criblages de la population, et en y rajoutant une répétition du test sur les individus présentant un virage sérique récent, il serait possible d'accroître le risque relatif (autrement dit "le pouvoir de prédiction") de plus de six fois, ceci comprenant tous les cas susceptibles de développer une lèpre multibacillaire; b) des niveaux élevés d'anticorps ont été détectés jusqu'à deux ans avant l'apparition clinique de la maladie, chez 70% des nouveaux cas; c) on a observé tant des virages asymptomatiques (élévation du titre) qu'un retour à la normale (chute du titre). Parmi 150 cas confirmés par biopsie, les résultats obtenus avec ELISA suggèrent que, dans la plupart des cas de lèpre paucibacillaire non compliquée, la chute du titre est rapide, se comptant en mois, mais que par contre dans les cas multibacillaires, elle était plus lente, s'étalant sur plusieurs années, ce qui reflète probablement la réponse au traitement, avec une augmentation du titre en cas de

réactivation. Ces résultats suggèrent que cette technique, menée avec un antigène amélioré, peut être utilisée dans les programmes de lutte contre la lèpre, tant pour repérer les individus qui pourraient bénéficier d'un traitement préventif, que poursuivre la réponse à la thérapeutique.

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