INTERNATIONAL JOURNAL OF LEPROSY

Volume 53, Number 4
Printed in the U.S.A.

Determination of Antibodies in Dried Blood from Earlobes of Leprosy Patients by Enzyme-linked Immunosorbent Assay—A Preliminary Report[1]

Wu Qin-xue, Ye Gan-yun, Zhou Li-lin, Shu Hui-wen, Liu Qi, Li Xin-yu, Ma Zhou-xiang, and Li Zhi-wen[2]

An important problem in the study of the epidemiology of leprosy is the lack of reliable technology for detecting subclinical infection and early serodiagnosis. It is well known that the FLA-ABS test first established by Abe ([1]) has been a useful tool for these purposes. Recently, however, a new, simple, rapid, sensitive and quantitative technology—enzyme-linked immunosorbent assay (ELISA)—has been developed and widely used to determine levels of human antibodies. We have measured antibody levels against *Mycobacterium leprae* in the blood of leprosy patients and healthy controls with an ELISA in our laboratory and our preliminary results are reported here.

MATERIALS AND METHODS

Blood samples

Group 1 blood samples consisted of 109 leprosy patients who were classified according to the Ridley-Jopling scale ([5]) (44 LL, 33 BL, 13 BB, 10 BT, and 9 TT). Group 2 consisted of samples from 101 individuals from a nonendemic area of leprosy. Group 3 was the standard positive serum (absorbance = 0.8 at 490 nm) and negative serum (absorbance = 0.2 at 490 nm) kindly provided by Dr. Young, Seattle, Washington, U.S.A., and positive and negative sera from native Chinese which we selected according to Dr. Young's criteria.

Blood was collected from the earlobe with 0.05 ml capillary glass tubes, immediately absorbed onto a filter paper strip ([7]), allowed to dry at room temperature for 4 hr, and put into a small plastic bag. After sealing, the bag was stored at −21°C until use.

The dry blood was reconstituted by adding 0.05 ml of distilled water, shaking well overnight at 4°C, and then a suspension of 0.05 ml BCG and *M. vaccae* and 0.035 ml of diluent A (M. Abe, personal communication, 1979) were added. The tube was shaken well and incubated at 37°C for 30 min. After incubation, the tube was centrifuged at 10,062 × *g* for 5 min. The absorbed supernatant was used for testing.

Antigen

The antigen was prepared from a fresh human leproma containing numerous *M. leprae*; the bacilli were separated by Abe's technology ([1]). The precipitated bacilli were suspended in 0.05 ml of 0.05 M carbonate–bicarbonate buffer (CBB), pH 9.6. The number of acid-fast bacilli (AFB) in this crude suspension was determined by Cao-Wu's technique ([3]), and the suspension was diluted with the same buffer so that the count was adjusted to 1×10^8/ml. The suspension was then sonicated 3 times for 10 min each time (with microprobe at 7 μm, Ultrasonic Disintegrator, MSE Instrument, England). The sonicated preparation of *M. leprae* was centrifuged at 22,640 × *g* for 20 min at 4°C. The supernatant was used as the stock solution of antigen. Its optical density (OD) value was 0.76 at 280 nm.

ELISA

The ELISA used in this report was an indirect assay performed as follows:

Coating of plates. The sonicated antigen of *M. leprae* was diluted at 1:1000 with 0.05 M CBB (pH 9.6). For coating plates, the

[1] Received for publication on 15 January 1985; accepted for publication in revised form on 12 July 1985.

[2] Q. Wu, Research Associate; G. Ye, Deputy Director; L. Zhou, Technician; H. Shu, Visiting Doctor; Q. Liu, X. Li, Z. Ma, Technicians, Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, Jiangsu, People's Republic of China. Z. Li, Deputy Director, Institute for Prevention and Treatment of Dermatology, Hei Long Jiang Province, People's Republic of China.

Reprint requests to Dr. Ye.

diluted antigen was added at 0.2 ml/well to a polystyrene microtiter plate of 40 wells (made in China), covered, and incubated overnight at 4°C. The plates were then washed 3 times with 0.2 ml of PBS/Tween 20 (0.1 M KH_2PO_4 sol. 42 ml, 0.4 M Na_2HPO_4 sol. 23 ml, 4 M NaCl sol. 29.1 ml, Tween 20 0.5 ml mixed and distilled water added to a final volume of 1000 ml, pH 7.2) per well.

Blocking of nonspecific binding. For blocking nonspecific binding of antibodies, the coated plate was preincubated with a solution of 5% bovine serum albumin (BSA) in PBS at 37°C for 2 hr (0.2 ml/well). As a further precaution to inhibit nonspecific binding, blood and enzyme-conjugated secondary antibody (ECSA) for testing were diluted in the presence of 1% BSA. In order to get cheaper and more practicable blocking agents, 5% goat serum (GS) and 20% egg albumin (EA) were used, and 1% GS and 5% EA were used, respectively, to dilute blood and ECSA for testing.

Incubation of test blood. The BSA was removed from the plate and replaced by the blood specimen (patient/normal) diluted to 1:100, 1:200, 1:400, 1:800 in PBS/Tween 20 with 1% BSA (0.2 ml/well). The plate was incubated for 2 hr at 37°C, then washed 4 times with PBS/Tween 20. The tests using GS and EA as blocking agents were conducted in the same manner.

Incubation with ECSA. IgG fraction goat anti-human IgG + IgA + IgM (from Shanghai Institute of Parasitic Diseases, China) was diluted 1:1000 in PBS containing 1% BSA, and added to plates at 0.2 ml/well. After 2 hr at 37°C, the plates were washed 3 times with PBS/Tween 20.

Color development. O-Phenylenediamine 20 mg was dissolved in 50 ml of citrate phosphate buffer (pH 5.0). Then 0.075 ml of 30% H_2O_2 was added and mixed. The substrate was added to the plate at 0.2 ml/well. After incubation in the dark at 37°C for 30 min, the reaction was stopped by the addition of 4 N sulfuric acid (0.05 ml/well) and color development was measured by absorbance at 490 nm using a DG-1 spectrophotometer.

Calculation of results. In each set of experiments, the reference positive and negative sera provided by Dr. Young or those selected by us were included as controls. Background levels of absorbance with PBS being substituted for serum were subtracted from each serum sample.

Statistical analysis. The upper limits for normal values were calculated with the method of percentiles ([4]) and termed theoretical normal values (TNV). For the purposes of application, the TNV was arbitrarily raised slightly, based on the combination of statistical principle and the actual results of tests, and termed the practical normal value (PNV). Additionally, Wolters' formula ([6]) was also used to determine normal values in this report.

RESULTS

The results are shown in The Table and The Figure. The Table shows the mean absorbance values and the percentage of positive reactions at four different dilutions of blood from 109 cases of leprosy and 101 normal controls, and the normal values which were obtained according to Wolters' formula ([6]) and the method of percentiles ([4]). The highest antibody levels are seen in BL/LL leprosy patients. The antibody activity in BT/TT leprosy patients was less pronounced than that in BL/LL patients. The BL patients had higher antibody activity than the BT/TT patients. Clearly, the mean OD values gradually increased from TT to LL. The normal controls had strikingly low antibody activity against *M. leprae*.

The positivity rates differed at the four different dilutions of blood. The positivity rate was highest at the 1:200 dilution where it was 66.6% in TT patients, and 100% in other types of patients. The mean positivity rate was 97.2% for the patients as a whole. The normal controls had a positivity rate of 4.9%.

Further analysis of the results at the 1:200 dilution of blood is shown in The Figure. There are wide variations in antibody content in blood from individual patients with similar clinical classifications, especially in BL/LL patients. The antibody content shows considerable overlap between the leprosy group and the normal control group at 0.4–0.7 OD values. The antibody content shows considerable overlap between the TT group and the normal

group at 0.2–0.7 OD values. The OD values of the majority of the normal control group are below 0.2.

DISCUSSION

One of the major problems in serologic testing is defining normal values. From the present results of our ELISA in leprosy patients at a 1:200 blood dilution the following definitions of normal were derived. According to Wolters' formula: P/N = E(test sample) − E(blank)/E(negative) − E(blank). A blood sample with a P/N value of 2.1 would be considered as positive. From the data in The Table, in our test, the upper limit of the normal value would be 0.15 × 2.1 = 0.31.

As calculated according to the method of percentiles with the following formula:

$$P_x = L + i/f_x(nx/100 - C_l)$$

From the results in The Table, $P_x = P_{95}$, L = 0.5, i = 0.1, $f_{95} = 2$, x = 95, n = 101, and $C_l = 98$. Substituting in the formula, the theoretical normal value (TNV) can be calculated to be 0.37 at the 1:200 dilution of the blood samples. We arbitrarily increased the value of the TNV (0.37) to 0.40 and termed this value the practical normal value (PNV).

As shown in The Figure, the upper limit for normal values obtained with Wolters' formula are lower than the upper limit of normal values obtained by the method of percentiles which are, in turn, lower than our practical normal values. The number of presumed false-positives among the normal control population progressively increases as the upper limits for normal decrease.

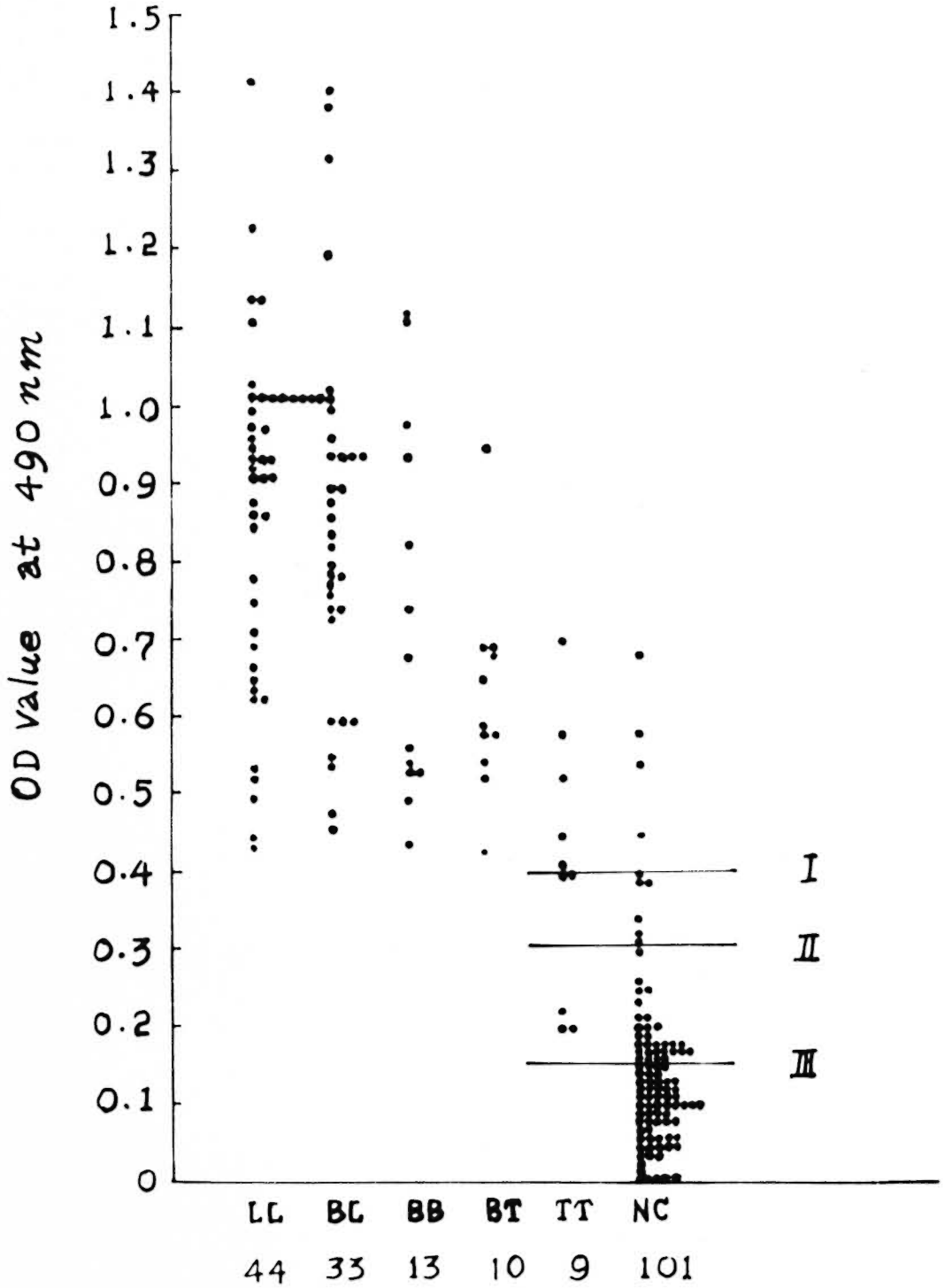
Another problem with the use of ELISA for serodiagnosis is standardization of the technique. For utility, the technique must have high sensitivity and high specificity. Brett, *et al.* ([2]) determined the optimum dilutions of sera and conjugate so as to yield the lowest number of false-positive results. From the results in The Table, our best results seemed to be obtained at a 1:200 dilution of blood, although this yielded a 4.9% presumed false-positive rate.

It is difficult to determine the significance of overlap in antibody content between normal individuals and leprosy patients. The antibody content of some normal individ-

THE TABLE. *ELISA results in leprosy cases and normal controls.*

	No. cases	Blood dilution							
		1:100		1:200		1:400		1:800	
		M_{OD}[a]	No. positive reactions	M_{OD}	No. positive reactions	M_{OD}	No. positive reactions	M_{OD}	No. positive reactions
Leprosy cases									
TT	9	0.64	4 (44.4)[b]	0.43	6 (66.6)	0.25	3 (33.3)	0.19	5 (55.6)
BT	10	0.80	7 (70.0)	0.62	10 (100.0)	0.59	9 (90.0)	0.58	9 (90.0)
BB	13	0.87	11 (84.6)	0.72	13 (100.0)	0.58	13 (100.0)	0.44	9 (69.2)
BL	33	0.92	28 (84.6)	0.84	33 (100.0)	0.68	32 (96.9)	0.53	30 (90.9)
LL	44	0.94	39 (88.6)	0.89	44 (100.0)	0.76	43 (97.7)	0.61	42 (95.4)
Total	109		89 (81.6)		106 (97.2)		100 (91.7)		95 (87.2)
Controls	101	0.31	5 (4.9)	0.15	5 (4.9)	0.10	1 (0.9)	0.06	1 (0.9)
Normal value		0.70[c] (0.67)[d] 0.65[c]		0.40 (0.37) 0.32		0.28 (0.25) 0.21		0.20 (0.18) 0.13	

[a] M_{OD} is the mean absorbance value at 490 nm.
[b] The numbers in parentheses refer to the positivity rate (%).
[c] The practical normal values (PNV).
[d] The theoretical normal values obtained with the method of percentiles.
[e] The normal value obtained according to Wolter's formula.



THE FIGURE. Distribution of antibody level in LL, BL, BB, BT, and TT leprosy patients at 1:200 dilution of the blood specimen. Each point represents one individual. Antibody level is expressed as OD value at 490 nm. Bar I = PNV; Bar II = normal values obtained with Wolters' formula; Bar III = M_{OD} value obtained from the normal control group.

uals overlapped with that of LL, BL, BB, and BT patients at OD values between 0.4 and 0.7. Similarly, there was overlap between normal individuals and TT patients in the range of 0.2 to 0.4 OD values. The majority of OD values in blood samples from normal persons was below 0.2. The overlap between normal individuals and leprosy patients could be due to subclinical infections among some normal individuals, crossreacting immune responses in some normal persons, or the loss of antibody responses in patients under treatment. From the present data, it might be advisable to consider OD values between 0.2 and 0.4 to be in a doubtful range.

Serologic evaluations have a number of possible uses in following leprosy patients. The ELISA has proved to be a sensitive technique to study humoral responses in mycobacterial diseases. Its specificity for *M. leprae* is enhanced by absorption of the blood with BCG, *M. vaccae,* cardiolipin, and lecithin according to Abe's technique. The present method is simple, rapid, requires little antigen, and provides at least a semiquantitative measurement. The technique may be useful as an epidemiological tool to detect subclinical leprosy infection and in evaluating contacts. Because the test can reflect antibody content, it is possible that it may be useful for following the response of patients to treatment, evaluating the status of disease among patients, and even estimating the degree of infectivity of leprosy patients.

SUMMARY

An enzyme-linked immunosorbent assay (ELISA) was used with soluble antigens of *Mycobacterium leprae.* All blood samples collected from the earlobes of 109 leprosy patients and 100 healthy controls (from a non-endemic area of leprosy) were absorbed with *M. vaccae,* BCG, cardiolipin, and lecithin according to the technology of the FLA-ABS test before being tested in the ELISA. The results (at a 1:200 blood dilution) showed that antibody activity gradually increased from TT to LL (mean OD values: TT = 0.43, BT = 0.62, BB = 0.72, BL = 0.84, LL = 0.89), and the rates of positive reactions were 100% in all classifications of patients except TT (66.6%). Antibody activity in the controls was less pronounced than in leprosy patients, their mean OD value being only 0.15. We suggest that the ELISA is highly sensitive and specific for the determination of anti-*M. leprae* antibodies, and is useful for clinical serodiagnosis and for the study of subclinical infections in leprosy.

RESUMEN

Se realizó un ensayo inmunoenzimático (ELISA) usando antígenos solubles del *Mycobacterium leprae.* Las muestras de sangre colectadas del lóbulo de la oreja de 109 pacientes con lepra y de 100 individuos sanos (de un área no endémica) se absorbieron con *M. vaccae,* BCG, cardiolipina, y lecitina, de acuerdo a la metodología para le prueba FLA-ABS antes de probarse por ELISA. Los resultados (a una dilución 1:200 de la sangre) mostraron que la actividad de anticuerpo aumentó gradualmente de TT a LL (valores medios de DO: TT = 0.43, BT = 0.62, BB = 0.72, BL = 0.84, LL = 0.89) y el grado de reacciones positivas fue del 100% en todos los grupos de pacientes excepto en los TT (66.6%). La actividad de anticuerpo en los controles fue menos marcada que en los pacientes con lepra, su DO media fue 0.15. Sugerimos que el ELISA es altamente sensible y específico para la determinación de anticuerpos anti-*M. leprae* y que es útil para el serodiagnóstico clinico y para el estudio de las infecciones subclínicas de le lepra.

RÉSUMÉ

Une épreuve ELISA (titrage avec immuno-absorbant lié à une enzyme) a été utilisé pour étudier les antigènes solubles de *Mycobacterium leprae.* Des échantillons de sang récoltés au niveau des lobules de l'oreille chez 109 malades de la lèpre et chez 100 témoins en bonne santé, ces derniers provenant d'une région indemne de lèpre, ont été absorbés avec *M. vaccae,* le BCG, la cardiolipine, et la lecithine, selon la technique des épreuves d'anticorps fluorescents (FLA-ABS), pour être ensuite étudiés par ELISA. Les résultats, à une dilution sanguine de 1:200, ont révélé une activité en anticorps qui augmentait progressivement tout au long du spectre clinique de la lèpre, des formes TT aux formes LL (moyenne des valeurs OD: TT = 0,43; BT = 0,62; BB = 0,72; BL = 0,84; LL = 0,89). Les taux de réactions positives s'élevaient à 100% dans chacun des groupes de malades, quel que soit le type clinique, sauf les TT (66,6%). L'activité en anticorps chez les sujets témoins était moins prononcée que chez les malades de la lèpre, leur valeur OD moyenne se situant à 0,15 seulement. On suggère dès lors que l'épreuve ELISA est hautement sensible et spécifique pour la détermination des anticorps contre *M. leprae,* et qu'elle est utile pour le sérodiagnostic clinique et pour l'étude des infections infra-cliniques dans la lèpre.

Acknowledgments. We would like to thank Dr. Abe for supplying the *M. vaccae,* BCG, cardiolipin, and lecithin, and Dr. Young for supplying the standard positive and negative sera.

REFERENCES

1. Abe, M., Minagawa, F., Yoshino, Y., Ozawa, T., Saikawa, K. and Saito, T. Fluorescent leprosy antibody absorption (FLA-ABS) test for detecting subclinical infection with *Mycobacterium leprae.* Int. J. Lepr. **48** (1980) 109–119.
2. Brett, S. J., Draper, P., Payne, S. N. and Rees, R. J. W. Serological activity of a characteristic phenolic glycolipid from *Mycobacterium leprae* in sera from patients with leprosy and tuberculosis. Clin. Exp. Immunol. **52** (1983) 271–279.
3. Department for Leprosy Research, Institute for Prevention and Treatment of Dermatology, Jiangsu Province, China. Survey of acid-fast bacilli in normal mice used for experiment. J. Prevent. Therap. Invest. Dermatol. **3** (1975) 311–317. (in Chinese)
4. Medical Institute of Si Cuan, China. *Medical Statistics.* 1978, pp. 96–103.
5. Ridley, D. S. and Jopling, W. H. Classification of leprosy according to immunity. A five-group system. Int. J. Lepr. **34** (1966) 255–273.
6. Samuel, N. M. and Adiga, R. B. Detection of antibodies in sera of leprosy patients and contacts by enzyme-linked immunosorbent assay (ELISA). Jpn. J. Lepr. **53** (1984) 32–37.
7. Wu, Q. X., Ma, Z. X., Shu, H. W., Zhou, L. L., Liu, Q., Ye, G. Y., Wang, C. J. and Ma, B. K. Comparison of FLA-ABS test employing sera from venous blood and blood from earlobes of 79 cases of leprosy. Acta Acad. Med. Sin. 7 (1985) 69–71.