INTERNATIONAL JOURNAL OF LEPROSY Volume 34, Number 4
Printed in U.S.A.

Card Tests for Leprosy[1]

Joseph Portnoy[2]

It has been estimated by the World Health Organization that there are 12 to 15 million patients with leprosy throughout the world. Most of the cases occur in the tropics. In the Americas the disease is reported in almost all countries, being highly prevalent in South America and to a lesser extent in Central America (20). Almost half of the cases in the Western world have been classified as lepromatous. However, because case finding is often inadequate, particularly in rural areas, the prevalence data are certainly minimal figures.

According to Canizares (2) the diagnosis of leprosy is based on at least three elements: (1) finding *M. leprae* in a smear made from an appropriate site; (2) alteration of neural sensitivity and sweat function, and (3) histologic examination.

Although advances have been made in the culture of *M. leprae* (3, 10, 18) and in treatment (2, 3, 8) of the disease, there is no serologic test for its diagnosis. It has been observed that positive reactions in serologic tests for syphilis occur to a fairly high degree in the lepromatous form of the disease (2, 6, 11, 12). A recent report suggests the possibility that double diffusion in gels may be useful in characterizing leprosy sera (7).

The Rapid Plasma Reagin (RPR) card test for syphilis and other treponematoses (13) was developed to provide a test that could be performed in the field or office without the need for equipment such as centrifuge, water bath, rotating machine, and microscope. Its effectiveness under field or clinic conditions has been confirmed (1, 19). Since the prevalence of the disease in rather remote areas suggested the value of this type of test, studies were undertaken with the hope of developing a test with a high capacity for detecting leprosy.

A number of observations suggested the feasibility of developing a test for leprosy. Ogata (9) reported that kaolin particles sensitized with cardiolipin and lecithin in a ratio of 1:1 were agglutinated more strongly by leprous sera than by syphilitic sera. On the other hand, greater agglutination of the particles sensitized with cardiolipin and lecithin in a ratio of 1:10 occurred with syphilitic sera than with leprous sera. Kent *et al.* (4, 5) noted a greater tendency for leprosy sera to react with antigens used in serologic tests for syphilis, but with a lower concentration of lecithin in those antigens. Schmidt (16), using complement fixation, found that antigen containing cardiolipin and cholesterol gave consistently higher titers with leprosy sera than antigens containing cardiolipin, lecithin, and cholesterol. Portnoy (12) observed a difference in the behavior of leprosy sera as compared to syphilis sera with respect to the presence of choline chloride in the antigen suspension, the reactions occurring with leprosy sera being inhibited by choline chloride while those with syphilitic sera were not.

The present report furnishes data pertaining to the development of card tests for leprosy for field use, in which hand shaking is used and for large scale testing in which a mechanical rotar is employed.

MATERIALS AND METHODS

Antigens. Stock antigen solutions in absolute alcohol were compounded from alco-

[1]Received for publication May 5, 1966.

[2]Joseph Portnoy, Ph.D., Director, Biological Research Laboratories, Hynson, Westcott and Dunning, Inc., Charles and Chase Streets, Baltimore, Maryland 21201. With the technical assistance of Susan Forester.

TABLE 1. *Results of VDRL type testing of leprosy antigens.*

Antigen No.	Ratio of cardiolipin to lecithin (w/w)	Serum A	Serum B	Serum C
1	1:3	N	N	N
2	1:2	N	N	N
3	1:1	N	N	N
4	1.5:1	N	N	N
5	5:1	N	N	N
6	7.5:1	N	N	N
7	No lecithin	N	N	N
VDRL slide[a]	1:7	R (1:8) (Titer)	WR	N

[a] Regular VDRL slide test antigen.
R = Reactive
WR = Weakly reactive
N = Nonreactive
Serums A and B were prepared by diluting a reactive serum (VDRL) pool in nonreactive Serum C.

holic solutions of cardiolipin (beef heart) and lecithin (beef heart or egg). The cholesterol was ash-free, reprecipitated from alcohol. All reagents had been found satisfactory for use in compounding antigens used in the serodiagnosis of syphilis. In some instances antigen solutions were made by mixing appropriate proportions of alcoholic solutions of the lipids.

Preparation of antigen suspension. The method was essentially similar to that employed in preparing antigen suspension for the RPR card test ([13]). The first step is similar to that used in preparing Venereal Disease Research Laboratories (VDRL) slide antigen suspension ([17]); where indicated the suspensions were used in this form for VDRL type testing. The antigen suspensions were then centrifuged at 2,000 times the force of gravity and the sediments were resuspended in a volume of suspending fluid equal to that of the suspension centrifuged and having the following composition:

Ethylenedinitrilotetraacetic acid (disodium salt) 0.25M pH 7.0	0.5 ml.
Phosphate buffered saline pH 6.4 containing 0.118 merthiolate	8.5 ml.
Charcoal suspension, aqueous (0.25%)	1.0 ml.

Serums. Leprosy serums were furnished by Dr. John R. Trautman, Chief, Clinical Branch, U. S. Public Health Service Hospital, Carville, Louisiana, whose cooperation is gratefully acknowledged. Clinical diagnoses, supplied by Dr. Trautman, were of either lepromatous or dimorphous leprosy. Qualitative VDRL tests had been performed at Carville and those specimens that were reactive (lepromatous type only) had a nonreactive (TPI) test. Serum samples were obtained also from the Maryland State Department of Health, whose cooperation is likewise acknowledged. These were routine specimens submitted for serologic tests for syphilis.

Leprosy card tests. These were carried out in a manner similar to the technic previously described for the RPR card test ([11, 14, 15]). Briefly, for the "circle" card tests, when the 18 mm. circle was used, 0.05 ml. of serum was spread on the plastic-coated card and 1/60 ml. of antigen suspension containing charcoal was added. Rotation was for 8 minutes at 100 rpm (mechanical rotator circumscribing a ¾ inch circle) and tests were read as "reactive" when clumping was noted or "nonreactive" when no clumping was seen. When the 14 mm. circle was employed, 0.03 ml. of serum was tested with 0.01 ml. of antigen suspension. When the "teardrop" surface was used, 0.03 ml. of serum was tested with 1/66 ml. of antigen suspension. Hand rocking was employed for 4 minutes, after which results were read. Quantitative tests were carried out by preparing dilutions in 0.9 per cent saline directly on the circle cards. When titers exceeded 1:32 dilutions were made in 1:50

nonreactive human serum to facilitate spreading of the serum dilutions.

RESULTS

Alcoholic solutions were prepared, containing constant concentrations of cholesterol (0.9%), cardiolipin (0.15%), and varying concentrations of lecithin (0-0.45%). VDRL type antigen suspensions were prepared and tested with the VDRL slide method against three control serums. The results obtained are given in Table 1. No evidence of reactivity was found, although the VDRL slide gave a 1:8 titer with serum A.

When these antigen suspensions were centrifuged and resuspended as described under MATERIALS AND METHODS, tests were made with syphilis and leprosy serums. Antigens 1 and 2 demonstrated some reactivity with syphilis serums, but the reactions were weaker than with the RPR card and VDRL slide tests. Antigens 3 and 4 showed promising behavior, being nonreac-

TABLE 2. *Results obtained with leprosy card test antigen suspensions containing varying ratios of cardiolipin to lecithin with serums from patients with syphilis or leprosy.*

	Experimental antigens used in circle card test[a]									
Serum	1	2	3	4	5	6	7	RPR card	VDRL[b] slide	Diagnosis
A	R	N	N	N	N	N	N	R	R	Serum
B	N	N	N	N	N	N	N	r	WR	in serum
C	N	N	N	N	N	N	N	N	N	controls
D	R	r	N	N	N	N	N	R	R	Serum in saline
E	r	r	N	N	N	N	N	R	R	controls, repre-
F	r	r	N	N	N	N	N	R	R	senting serial
G	r	r	N	N	N	N	N	R	WR	two-fold
H	N	N	N	N	N	N	N	N	N	dilutions
L 1	N	N	N	N	N	N	N	N	N	Lepromatous
L 2	4[(a)]	8	16	32	N	N	N	N	N	"
L 3	4	16	32	64	N	N	N	N	N	"
L 4	2	8	16	64	1	1	1	N	N	"
L 5	N	2	2	4	1	1	N	N	N	"
L 8	2	8	16	32	N	N	N	N	R	"
L 9	8	16	256	512	N	N	N	N	R	"
L 10	2	8	16	64	1	N	N	N	R	"
L 11	4	16	32	64	1	1	1	N	R	"
L 12	8	64	256	512	N	N	N	N	R	"
L 13	8	16	64	256	1	1	1	N	R	"
L 14	4	8	32	64	1	N	N	N	R	"
L 15	N	2	4	4	1	1	1	N	N	Dimorphous
L 16	N	N	N	N	N	N	N	N	N	"
L 17	N	N	N	N	N	N	N	N	N	"
L 18	N	N	N	N	N	N	N	N	N	"
L 19	N	N	N	N	N	N	N	N	N	"
L 20	N	N	N	N	N	N	N	N	N	"
L 21	N	N	N	N	N	N	N	N	N	"
L 22	N	N	N	N	N	N	N	N	N	"

[a]Antigens same as in Table 1.
[b]As reported from Carville Public Health Service Hospital.
Serums A, B, and C same as in Table 1.
Serums D-H represent dilution of a single syphilis serum in saline.
R = Reactive
r = Minimally reactive
N = Nonreactive
WR = Weakly reactive
[(a)] = Reciprocal of highest dilution giving a reactive or minimally reactive result.
L = Leprosy

TABLE 3. *Effect of varying both the ratio of cardiolipin to lecithin and absolute concentration of these lipids on reactivity with syphilis and leprosy serums.*

Antigen No.	% cardiolipin	Ratio cardio/lec	A	B	C	D	E	F	G	H	L PP	L NP
8	.1	1:3	r	N	N	R	R	R	R	N	16	N
9	.05	1:3	r	N	N	R	R	R	R	N	8	N
10	.038	1:3	r	N	N	r	r	r	r	N	8	N
11	.1	1:2	N	N	N	r	r	r	r	N	16	N
12	.05	1:2	N	N	N	r	r	r	N	N	16	N
13	.038	1:2	N	N	N	N	N	N	N	N	16	N
14	.1	1:1.9	N	N	N	r	r	r	r	N	32	N
15	.1	1:1.6	N	N	N	r	r	r	N	N	64	N
16	.1	1:1.3	N	N	N	r	N	N	N	N	64	N
17	.2	1:1	N	N	N	R	r	N	N	N	64	N
18	.15	1:1	N	N	N	R	r	N	N	N	128	N
19	.1	1:1	N	N	N	N	N	N	N	N	128	N
20	.05	1:1	N	N	N	N	N	N	N	N	64	N
21	.038	1:1	N	N	N	N	N	N	N	N	32[a]	N
22	.1	1:.9	N	N	N	N	N	N	N	N	128	N
23	.1	1:.8	N	N	N	N	N	N	N	N	128	N
24	.1	1:.7	N	N	N	N	N	N	N	N	128	N
25	.1	1:.6	N	N	N	N	N	N	N	N	128	N
26	.1	1:.5	N	N	N	N	N	N	N	N	128	N
RPR card			R	r	N	R	R	R	R	N	N	N
VDRL slide			R	WR	N	R	R	R	WR	N	WR	N

[a]Minimally reactive in all dilutions tested up to 1:32.
Cardio = Cardiolipin
Lec = Lecithin
A-H = See footnote, Table 2
LPP = Leprosy-positive pool
LNP = Leprosy-negative pool

tive with syphilis serum and giving a high degree of positivity with the serums of leprosy patients. These antigens contained a ratio of 1 to 1 and 1.5 to 1, respectively, of cardiolipin to lecithin. The concentration of cardiolipin was 0.15 per cent. Higher ratios of cardiolipin to lecithin were much less reactive, and in the absence of lecithin (antigen 7) only weak reactions were observed.

The area to concentrate upon having been found, additional antigens were prepared and the effect of changing the absolute concentrations of the lipids within certain ratios of cardiolipin was determined. Again it was noted that when the relative concentration of lecithin to cardiolipin was greater than 1:1 there was a tendency for antigens to react with syphilis serum and reduced reactivity with the leprosy pool occurred. When the cardiolipin concentration was greater than 0.1 per cent a slight tendency for reactivity with syphilis serum was noted. Antigens 19, 22, 23, 24, 25, and 26 containing 0.1 per cent cardiolipin, with ratios of cardiolipin to lecithin of 1 to 1 up to 2 to 1 gave similar titers with the leprosy-positive pool.

From this large number of antigens, five were selected for the testing of individual leprosy serums. The results are presented in Table 4. At this time VDRL slide tests also were performed. Although the reactivity in the VDRL slide test was barely evident, as compared to the findings reported from Carville, intense reactivity was observed with these selected card antigens. Antigen 26 tended to give the highest titers, although the intensity of the clumping tended to be greater with antigen 19.

To determine the capacity of antigens designed for leprosy to react with nonleprosy serums, a limited number of specimens were examined which had been submitted to the Maryland Department of

Health for serologic tests for syphilis. The results are given in Table 5. Of 26 specimens that were nonreactive in the RPR card test, two specimens reacted with all three leprosy antigens with a titer of four. Of 22 specimens that were reactive in the RPR card test, only one specimen reacted with the leprosy antigens, giving a titer of 16.

Tests using the tear-drop surface were carried out with the use of antigens described in Table 5, and with results similar to those obtained with the circle card.

DISCUSSION

The results of this investigation point to the superiority of antigens containing a ratio of cardiolipin to lecithin of 1:1 up to

TABLE 4. *Comparative reactivity of selected leprosy antigens with the use of individual sera.*

Leprosy serum	Ratio, cardio/lec— antigen number—	1:1.6 15	1:1.3 16	1.1 19	1:.7 24	1:.5 26	VDRL slide
L 1		r	r	N	N	N	N
L 2		16	32	32	16	16	N
L 3		64	64	64	128	256	N
L 4		32	64	64	64	64	N
L 5		8	16	8	4	8	N
L 9		128	128	256	256	1024	1
L 11		64	64	128	128	128	N
L 13		128	256	256	256	256	1
L 14		64	64	64	128	256	N
D 15		8	16	8	8	16	WR
D 16		N	N	N	N	N	N
D 17		N	N	N	N	N	N
D 18		N	N	N	N	N	WR
D 19		N	N	N	N	N	N
D 20		N	N	N	N	N	N
D 21		N	N	N	N	N	N
D 22		N	N	N	N	N	Not done

Cardio = Cardiolipin
Lec = Lecithin
L = Lepromatous
D = Dimorphous

TABLE 5. *Reactivity of 3 selected leprosy antigens with nonleprosy serum.*

RPR card qual.	VDRL slide quant.	Number of specimens	28 Cardio. 1% Lec. 1%	30 Cardio. 1% Lec. .075%	29 Cardio. 1% Lec. .05%
N	Not done	24	N	N	N
N	Not done	2	4	4	4
R	1	4	N	N	N
R	2	2	N	N	N
R	2	1	16	16	16
R	4	2	N	N	N
R	8	3	N	N	N
R	16	2	N	N	N
R	32	1	N	N	N
R	64	6	N	N	N
R	128	1	N	N	N

a ratio of 2:1 in detecting evidence of serologic reactivity in leprosy, essentially confirming Ogata's report ([9]). Such antigens show a markedly reduced capacity to react with syphilis serum, whereas antigens containing a ratio of cardiolipin to lecithin of 1 to 7 or higher have a greater capacity to react with syphilis serums. The tendency of antigens used in the serodiagnosis of syphilis to react with leprosy serum, particularly from patients with lepromatous type disease, appears to be much greater than the tendency of the antigens designed for use with leprosy to react with syphilis serums. Another important difference in the behavior of the lipid-reactive antibodies in leprosy and syphilis is the observation that choline chloride inhibits the reactivity of leprosy serums and tends to enhance the reactivity of syphilis serums ([12]). The greater specificity of the RPR card test as compared to the VDRL slide test with leprosy serums depends upon this phenomenon ([1, 13]). This study indicated that the leprosy antigens reacted to only a slight degree with nonleprosy serums and there appeared to be no correlation of this reactivity with syphilitic serums.

Matthews and Trautman ([6]) indicated that removal of the cryoprotein fraction of leprosy sera brought about a nonreactive VDRL slide test result in specimens previously reactive. The findings obtained in this report suggest that the reactivity observed with the leprosy antigens was not associated with cryoproteins. Although there was a distinct reduction in the reactivity of the VDRL slide test (compare Table 1 and Table 4), as obtained in this laboratory as compared with the results reported from Carville, the reactivity with the leprosy antigens remained high in spite of the fact that specimens had been under study for four months and had been filtered to remove particulate matter.

The attributes of the card test should make it useful for field and clinic conditions, particularly in remote areas in developing countries where the need is greatest. Whether the sensitivity of the card test for leprosy is adequate or not, remains to be determined by further studies. The observation of fairly high titers in four out of five lepromatous cases found to be nonreactive in the VDRL slide test, and in one out of seven dimorphous cases, is encouraging.

SUMMARY

The principle of the card test, which employs antigens containing charcoal in an agglutination reaction with unheated serum or plasma on a plastic-coated surface, has been adapted as a test for leprosy. Antigen suspensions containing cardiolipin and lecithin in ratios between 1 to 1 and 2 to 1 appeared promising.

RESUMEN

El principio de prueba en tarjeta, a base de antígenos conteniendo carbón en una reacción de aglutinación con suero o plasma no calentado en una superficie cubierta de materia plástica, se ha adaptado como una prueba para lepra. Suspensiones de antígeno conteniendo cardiolipina y lecitina en proporción entre 1 a 1 y 2 a 1 parecen tener un futuro promisorio.

RÉSUMÉ

On a adapté le principe de l'épreuve sur carte pour mettre au point une épreuve pour la lèpre. Ce principe est basé sur l'emploi d'antigènes contenant du charbon de bois pour développer une réaction d'agglutination avec du sérum ou du plasma non-chauffés sur une surface revêtue de matière plastique. Il semble que des suspensions d'antigènes contenant de la cardiolipine et de la lécithine, dans une proportion de 1 : 1 du de 2 : 1, soient prometteuses.

REFERENCES

1. Buck, A. A. and Mayer, H. Comparative studies of the rapid plasma reagin card test for syphilis and VDRL slide test in Ethiopia. American J. Hyg. **80** (1964) 85-90.
2. Canizares, O. Diagnosis and the treatment of leprosy in the United States. Med. Clin. N. America **49** (1965) 801-816.

3. [Closing in on leprosy.] Medical World News **6** (1965) 27-29 (June 4).
4. KENT, J. F., OTERO, A. G. and HARRIGAN, R. E. Relative specificity of serologic tests for syphilis in *M. leprae* infection. American J. Clin. Path. **27** (1957) 539.
5. KENT, J. F., BURKE, J. C., CARROLL, D. P., SIMONTON, L. A. and OTERO, A. G. Differentiation of the antilipids occurring in nontreponemal diseases and syphilis. J. Chron. Dis. **7** (1958) 36-42.
6. MATTHEWS, L. J. and TRAUTMAN, J. R. Clinical and serologic profile in leprosy. Lancet **2** (1965) 915-918.
7. NAVALKAR, R. G., NORLIN, M. and OUCHTERLONY, O. Characterization of leprosy sera with various mycobacterial antigens using double diffusion in gel analyses. Internat. Arch. Allergy & Appl. Immunol. **28** (1965) 250-260.
8. [New therapies for leprosy.] Medical World News **22** (1966), p. 41, p. 61 (Feb. 4).
9. OGATA, T. A new serological property of leprous sera revealed by cardiolipinlecithin antigen. Atti VI Congr. internaz. Microbiol. (Rome) **2** (1953) 183.
10. OLITZKI, A. L. Maintenance of cytopathic activity of *M. leprae* in Eagle's medium supplemented by mycobacterial extracts. Israel J. Med. Sci. **1** (1965) 1004-1008.
11. FORTNOY, J., GALVEZ, R. and CUTLER, J. C. Clinical and serologic studies with reference to syphilis in Guatemala, Central America. III. Studies of comparative performance of the Kahn, Kolmer, Mazzini, and VDRL slide test among leprosy patients. American J. Syph. **36** (1952) 566-570.
12. PORTNOY, J. and EDMUNDSON, W. F. A simple procedure for the identification of nonsyphilitic reactions in serologic tests for syphilis in leprosy patients. Internat. J. Leprosy **22** (1954) 181-194.
13. PORTNOY, J. BREWER, J. H. and HARRIS, A. Rapid plasma reagin card test for syphilis and other treponematoses. Pub. Hlth. Rep. **77** (1962) 645-652.
14. PORTNOY, J. Modifications of the rapid plasma reagin (RPR) card test for syphilis for use in large scale testing. American J. Clin. Path. **40** (1963) 473-479.
15. PORTNOY, J. A note on the performance of modifications of the rapid plasma reagin (RPR) card test for syphilis, for use in large scale testing. Pub. Hlth. Lab. **23** (1965) 43.
16. SCHMIDT, H. Reactivity of a lecithin-free cardiolipin preparation (cardchoe) in leprosy sera. Bull. WHO. **20** (1959) 1175.
17. [Serologic tests for syphilis, 1964 Manual.] Pub. Hlth. Serv. Publ. No. 411 (Revised May 1964), U. S. Government Printing Office, Washington, 1964.
18. SHEPARD, C. C. Leprosy bacilli in mouse foot pads. *In* Pathogenesis of Leprosy, Ciba Foundation Study Group No. 15. G. E. W. Wolstenholme and M. O'Connor, Eds. Boston, Little, Brown & Co., 1963, pp. 80-88.
19. TIEDEMANN, J. H. and MULLINS, J. The RPR card test as a diagnostic aid in a venereal disease clinic. Pub. Hlth Lab. **24** (1966) 12.
20. [WORLD HEALTH ORGANIZATION] Chronicle. Notifiable diseases in the Americas. **20** (1966) p. 50.