Serological Activity of Natural Disaccharide Octyl Bovine Serum Albumin (ND-O-BSA) in Sera from Patients with Leprosy, Tuberculosis, and Normal Controls[1]

Wu Qin-Xue, Ye Gan-Yun, and Li Xin-Yu[2]

There have been many reports of studies on the serological activity of whole *Mycobacterium leprae* (WML) and the *M. leprae*-specific phenolic glycolipid-I (PGL-I) antigen from *M. leprae* ([3, 5, 6, 9]). There is crossreactivity of WML with other mycobacteria, and preliminary results in screening sera from healthy controls, tuberculosis patients, and leprosy patients have shown some crossreactivity of PGL-I with sera from a few normal controls and tuberculosis patients ([8]). Our results have shown the presence of similar crossreactivity ([9]). For the purpose of serodiagnosis for leprosy, investigators have to find newer and more specific antigens for detecting *M. leprae* infection. Natural disaccharide-octyl-bovine serum albumin (ND-O-BSA) antigen, the specific natural disaccharide of PGL-I (3,6 Me_2Glc-2,3 Me_2Rha) conjugated with bovine serum albumin (BSA) is a semi-synthetic antigen. Theoretically, it should be more specific for detecting infection with *M. leprae* than PGL-I.

We report here the results of some comparisons of ND-O-BSA with WML and PGL-I in the sera of leprosy patients, tuberculosis patients, and normal individuals.

MATERIALS AND METHODS

Blood samples. Blood samples were taken from 151 leprosy patients (classified according to the Ridley-Jopling classification as LL = 37, BL = 37, BB = 23, BT = 27, and TT = 27), 20 active tuberculosis patients, and 42 healthy individuals from an area which is nonendemic for leprosy.

Antigens. WML, PGL-I, and ND-O-BSA antigens were kindly provided by Dr. Patrick Brennan (Colorado State University, Fort Collins, Colorado, U.S.A.). They were coated by ourselves to 40-well, flat-bottom, polystyrene microtiter plates (Third Factory for Plastic Products, Shanghai, China). The WML suspension was prepared according to Douglas' method (Douglas, J. T., personal communication, 1986). The concentration for coating the plates was standardized with an optical density (OD) value (i.e., the OD value of WML suspension in 0.01 M acetate-carbonate buffer, pH 8.2, was 0.04 at 420 nm). The PGL-I suspension was reconstituted by the methods of Cho, *et al.* ([5]) and Young and Buchanan ([10]). The pure PGL-I in coating buffer (CB) (Na_2CO_3 0.398 g, $NaHCO_3$ 0.733 g, distilled water 250 ml, pH 9.6) was sonicated for 60 sec (with microprobe at 1.5 μm; Ultrasonic Disintegrator, MSE Instruments, England). A milky, stable suspension was obtained. This suspension was then diluted with CB to the required concentration (2.5 μg/ml) for coating the plates. Each vial of ND-O-BSA contained 50 μg of sugar (as glucose equivalent) and 200 μg of BSA, and represented 0.5 ml of lyophilized material. After adding 0.5 ml of distilled water for reconstitution, this solution was diluted with CB to the required concentration (0.1 μg/ml) for coating the plates.

Blocking agent. Skim milk (SM), an instant nonfat dry milk (Lucerne, brought back from Honolulu, Hawaii, July 1986) was dissolved with phosphate-buffered saline (PBS) (Na_2HPO_4 12.8 g, NaH_2PO_4 2.62 g, NaCl 0.58 g, distilled water 1000 ml, pH 7.4). Its working concentration was 2.5% w/v.

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[2] Q.-X. Wu, Associate Professor; G.-Y. Ye, Professor; X.-Y. Li, Research Assistant, Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, Jiangsu, People's Republic of China.

Reprint requests to Professor Ye.

Conjugates and color reagent. Horseradish peroxidase (HRP)-conjugated antihuman IgA, IgG, IgM, and IgAGM (DAKO; Accurate Chemicals, Westbury, New York, U.S.A.) were diluted with PBS containing 2.5% SM. Their working concentrations were all at 1:1000. o-Phenylenediamine (OPD; Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was dissolved in citrate buffer (citric acid 4.67 g, Na_2HPO_4 7.3 g, distilled water 1000 ml, pH 5.0) and diluted to the required concentration (0.04%).

ELISA. The enzyme-linked immunosorbent assay (ELISA) was essentially conducted with Dr. Brennan's procedures (Brennan, P. J., personal communication, 1986) which can be stated briefly as follows: 0.1 ml of WML, or PGL-I, or ND-O-BSA in coating buffer was added to each well of flat-bottom microtiter plates. Antigen-coated wells were blocked to prevent nonspecific binding by adding 0.2 ml of 2.5% SM in PBS (pH 7.4) and incubating at 37°C for 1 hr. After emptying the blocking agent, 0.1 ml of serum diluted 1:200 in PBS containing 2.5% SM was added to duplicate wells and incubated at 37°C for 1 hr. After washing four times with PBS, 0.1 ml of peroxidase-conjugated anti-human IgA, or IgG, or IgM, or IgAGM conjugate diluted (1:1000) in PBS containing 2.5% SM was added and incubated for 1 hr at 37°C. The plates were then washed four times with PBS, and 0.1 ml of substrate (0.003% hydrogen peroxide) and color reagent (0.04% o-phenylenediamine) in citrate buffer were added and incubated in the dark for 20 min at 37°C. The reaction was stopped by the addition of 0.05 ml of 2.5 N sulfuric acid. The results were read at 490 nm with a Model DG-3022 ELISA detector (Huadong Electric Tube Factory, Nanjing, China).

In the tests for selecting the optimum conditions for ND-O-BSA, the HRP-IgM was diluted into five different concentrations (1:1000, 1:2000, 1:4000, 1:6000, and 1:8000). The dilutions for each serum used were 1:20, 1:100, 1:300, 1:500, and 1:700. The concentrations of OPD were 0.01%, 0.02%, 0.03%, and 0.04%, and ND-O-BSA was diluted to 0.05 μg/ml, 0.1 μg/ml, 0.15 μg/ml, and 0.2 μg/ml. The conjugates and sera were diluted with PBS containing 2.5% SM, OPD with citrate buffer, and ND-O-BSA with CB. The optimum conditions for the ELISA were all the same as those mentioned above except for the concentration of ND-O-BSA, which was 0.1 μg/ml.

Statistical analysis. The upper limit for normal values was calculated as the mean plus three standard deviations of the values of the normal sera, and termed normal value (NV). For the purpose of practical application, however, the NV was raised, based on the combination of statistical principles and the actual results of the tests, and termed the practical normal value (PNV). Additionally, we have conducted correlation analyses among the results obtained with the antigens tested. Values of patients' sera/values of normal sera (P/N) ratios were used for comparisons of serological activity of leprosy sera in the WML, PGL-I, and ND-O-BSA ELISAs. The differences between values with patients' sera (P) and values with normal sera (N) (i.e., P − N) were also used.

RESULTS

Optimum conditions for ND-O-BSA ELISA. According to our tests for the ND-O-BSA ELISA, the best results were obtained when the following conditions were used: ND-O-BSA 0.1 μg/ml, OPD 0.04%, serum dilution at 1:200, and HRP-IgM dilution at 1:1000 (Table 1, Fig. 1).

Results of studies on serological activity in ELISA. When ELISA values from a total of 116 leprosy patients, 20 tuberculosis patients, and 30 normal controls were analyzed (Fig. 2), there was a significant correlation between the results with the WML and PGL-I antigens (correlation coefficient $r = 0.88$, $p < 0.0005$), and there was a significant correlation between the results with PGL-I and ND-O-BSA as well ($r = 0.86$, $p < 0.0005$). These results suggest that the antigenicity and specificity of WML, PGL-I, and ND-O-BSA in the sera tested are similar.

Table 2 shows the results of the WML, PGL-I, and ND-O-BSA ELISAs. In leprosy patients, the positivity rates were 100% in the PGL-I ELISA and 97.4% in both the WML and ND-O-BSA ELISAs. The positivity rate was 0% in all of the normal controls. But in the tuberculosis patients, the positivity rates were 95% with WML (mean OD = 0.24), 75% with PGL-I (mean OD = 0.11), and 65% with ND-O-BSA (mean

TABLE 1. *IgA, IgG, IgM, and IgAGM antibodies to ND-O-BSA in patient and normal sera (serum dilution 1:200).*

	No.	Results of ND-O-BSA ELISA (M_{OD})[a]			
		IgA	IgG	IgM	IgAGM
Patients					
LL	7	0.32 ± 0.16	0.58 ± 0.51	1.11 ± 0.30	2.44 ± 0.55
BL	7	0.15 ± 0.08	0.69 ± 0.50	0.78 ± 0.30	1.63 ± 0.77
BB	7	0.15 ± 0.08	0.78 ± 0.46	0.65 ± 0.17	1.59 ± 0.40
BT	7	0.09 ± 0.06	0.51 ± 0.22	0.51 ± 0.29	1.26 ± 0.54
TT	7	0.14 ± 0.07	0.56 ± 0.33	0.43 ± 0.22	0.96 ± 0.53
Average		0.17 ± 0.12	0.63 ± 0.35	0.70 ± 0.35	1.65 ± 0.76
Normal	12	0.04 ± 0.05	0.17 ± 0.08	0.04 ± 0.04	0.33 ± 0.10
P/N[b]		4.25	3.70	17.50	5.00
P_{TT}/N[c]		3.50	3.29	10.75	2.90
P − N[d]		0.13	0.46	0.66	1.32

[a] M_{OD} = mean optical density.
[b] P/N = M_{OD} (patient samples): M_{OD} (normal controls).
[c] P_{TT}/N = M_{OD} (TT patient samples): M_{OD} (normal controls).
[d] P − N = M_{OD} (patient samples) − M_{OD} (normal controls).

OD = 0.08). These data suggest that there is essentially the same sensitivity and specificity to the three antigens used, but that crossreactivity with *M. tuberculosis* was present at different levels. On the other hand, from TT to LL the content of antibody increased gradually with the three antigens used, and the content of antibody against the three antigens is negligible in normal controls.

Table 3 shows the results of the WML, PGL-I, and ND-O-BSA ELISAs when the definitions of the upper limit of the normal values are changed. From this table, we find that at normal value (a) (defined by the mean of the OD values of the normal controls plus three standard deviations), the reactivity of WML, PGL-I, and ND-O-BSA with *M. tuberculosis* is the same as shown in Table 2. At normal value (b) (defined arbitrarily as three times a), the rates of crossreactivity decrease in order of WML, PGL-I, and ND-O-BSA. At normal value (c) (defined arbitrarily as four times a), the rates of crossreactivity of PGL-I and WML with *M. tuberculosis* become 25% and 35%, respectively, and that of ND-O-BSA becomes 0%. Although crossreactivity of ND-O-BSA with *M. tuberculosis* disappears with these definitions, the rates of positivity do not decline

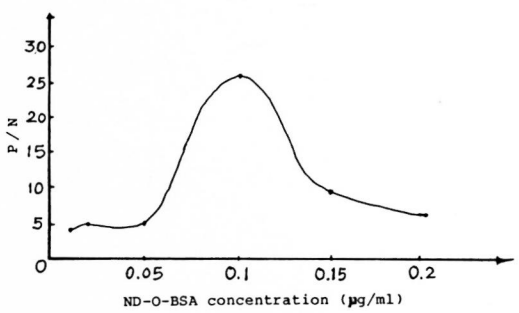

FIG. 1. P/N curve of ND-O-BSA antigen for selecting the optimum concentration. Horseradish peroxidase-IgM 1:1000; serum dilution 1:200; o-phenylenediamine concentration 0.04%.

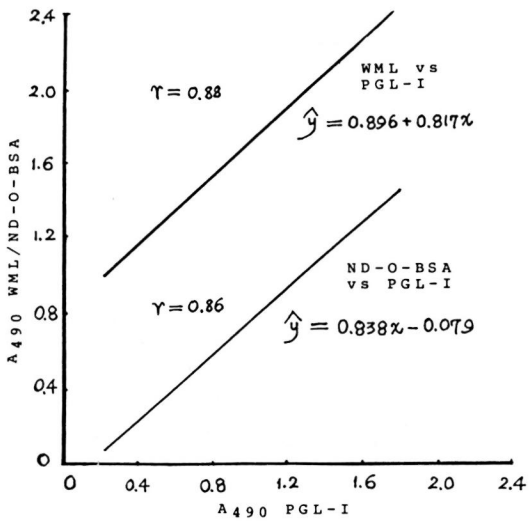

FIG. 2. Correlations among WML, PGL-I, and ND-O-BSA antigens in sera from patients with leprosy (116), tuberculosis (20), and normal controls (30).

TABLE 2. *Comparison of activites againt leprosy sera of whole* M. leprae *(WML), PGL-I, and ND-O-BSA (horseradish peroxidase-IgM 1:1000, serum dilution 1:200).*

	No.	No. (%) positive for			Mean A_{490} for		
		WML	PGL-I	ND-O-BSA	WML	PGL-I	ND-O-BSA
Patients							
LL	30	30 (100)	30 (100)	30 (100)	1.09 ± 0.61	1.16 ± 0.62	0.97 ± 0.86
BL	30	30 (100)	30 (100)	30 (100)	1.00 ± 0.53	1.04 ± 0.54	0.69 ± 0.69
BB	16	16 (100)	16 (100)	16 (100)	0.77 ± 0.48	0.88 ± 0.52	0.56 ± 0.32
BT	20	20 (100)	20 (100)	20 (100)	0.40 ± 0.22	0.37 ± 0.21	0.19 ± 0.09
TT	20	17 (85)	20 (100)	17 (85)	0.30 ± 0.27	0.37 ± 0.39	0.20 ± 0.18
Total	116	113 (97.4)	116 (100)	113 (97.4)			
Average					0.77 ± 0.56	0.80 ± 0.59	0.58 ± 0.65
TB[a]	20	19 (95)	15 (75)	13 (65)	0.24 ± 0.19	0.11 ± 0.08	0.08 ± 0.05
Normal	30	0	0	0	0.02 ± 0.02	0.006 ± 0.01	0.01 ± 0.01
P/N					38.50	133.33	58.00
Normal value[b]					0.07	0.04	0.05

[a] TB = tuberculosis patients.

[b] Normal value = M_{OD} of healthy controls + 3 S.D. Positive for WML was $0.02 + 3 \times 0.016 \cong 0.07$; for PGL-I = $0.006 + 3 \times 0.009 \cong 0.04$; for ND-O-BSA = $0.01 + 3 \times 0.012 \cong 0.05$.

significantly with leprosy patients, especially with multibacillary leprosy patients.

DISCUSSION

In this study, we established conditions for an ELISA based on ND-O-BSA and compared the WML, PGL-I, and ND-O-BSA ELISAs.

From the data in Figure 2, there are highly significant positive correlations among the WML, PGL-I, and ND-O-BSA ELISAs, which suggest that the antigenicity of the three antigens tested is similar. This conclusion is supported with results as shown in Table 2. All three methods were highly sensitive and specific with sera from recognized leprosy patients and normal controls, but there was crossreactivity with *M. tuberculosis.* The crossreactivity gradually declined in order of WML, PGL-I, and ND-O-BSA. These data suggest that the ND-O-BSA antigen is more specific for leprosy than the PGL-I and WML antigens. This specificity may depend on the molecular structures of these antigens.

Practical application

Evaluating the effectiveness of chemotherapy and status of disease among patients. Table 1 shows that mean OD values of IgM-, IgG-, and IgAGM-class antibodies gradually increased from TT to LL patients. The results from Table 2 also support the results from Table 1. These data suggest that mean OD values are related to bacterial load or bacterial index (BI). This agrees with the report of Schwerer, *et al.* ([7]). They found that there was a linear correlation between IgM anti-PGL-I antibody levels and BI, and that elevated anti-PGL-I IgM in bacillary-negative patients was usually indicative of active disease, undetected by BI. Hence, they concluded that anti-PGL-I IgM levels were valuable for monitoring the degree of disease activity. Our preliminary results (unpublished) support this. From the results shown in Tables 1 and 2, we conclude that WML, PGL-I, and ND-O-BSA all have the same practical significance for monitoring the effectiveness of chemotherapy and the degree of disease activity.

Immunoepidemiological studies of leprosy in the field. There have been many reports of the FLA-ABS test and the PGL-I ELISA being used as epidemiological tools to determine the prevalence of subclinical infection with *M. leprae* in high-risk populations ([1, 2]). These studies are very important in developing a strategy for control and eradication programs in leprosy. From our studies, the WML ELISA and the PGL-I ELISA may be used as tools for preliminary screening for *M. leprae* infection, but the ND-O-BSA ELISA is even more useful.

Use in serodiagnosis of leprosy. Abe ([1]) has suggested the combination of the FLA-ABS test and ELISA for the serodiagnosis

TABLE 3. *Analysis of sensitivity and specificity of WML, PGL-I, and ND-O-BSA ELISAs and their practical normal values (horseradish peroxidase-IgM 1:1000, serum dilution 1:200).*

	No.	No. (%) positive for ELISA using								
		WML			PGL-I			ND-O-BSA		
		0.07[a]	0.21[b]	0.28[c]	0.04[a]	0.12[b]	0.16[c]	0.05[a]	0.15[b]	0.20[c]
Patients										
LL	30	30 (100)	29 (96.6)	29 (96.6)	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)	29 (96.6)
BL	30	30 (100)	29 (96.6)	29 (96.6)	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)	29 (96.6)
BB	16	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)
BT	20	20 (100)	19 (95)	17 (85)	20 (100)	17 (85)	17 (85)	20 (100)	13 (65)	11 (55)
TT	20	17 (85)	9 (45)	8 (40)	20 (100)	12 (60)	9 (45)	17 (85)	8 (40)	7 (35)
Total	116	113 (97.4)	102 (87.9)	99 (85.3)	116 (100)	105 (90.5)	103 (88.7)	113 (97.4)	97 (83.6)	92 (79.3)
TB	20	19 (95)	9 (45)	7 (35)	15 (75)	9 (45)	5 (25)	13 (65)	1 (5)	0
Normal	20	0	0	0	0	0	0	0	0	0

[a] Represents theoretical upper limit of normal value (i.e., mean of the normal control sera OD readings plus 3 S.D.).
[b] Three times [a].
[c] Four times [a] (supposed practical normal value, PNV).

of leprosy. Chakinis, *et al.* ([4]) have pointed out that ELISA and RIA in combination were more reliable. From our studies, the combination of the PGl-I and ND-O-BSA ELISAs may be ideal for these purposes.

SUMMARY

We studied the natural disaccharide-octyl-bovine serum albumin (ND-O-BSA) enzyme-linked immunosorbent asay (ELISA) in sera from 151 leprosy patients, 20 tuberculosis patients, and 42 normal persons from a nonendemic area. The three ELISAs, whole *Mycobacterium leprae* (WML), phenolic glycolipid-I (PGL-I), and ND-O-BSA, are all highly sensitive for detecting antibodies against *M. leprae.* The results indicate that the serological activity has highly significant, positive correlations among the three types of antigens used. Their positivity rates are 100% with PGL-I and 97.4% with WML and ND-O-BSA in leprosy patients, and 0% with any antigen used in normal persons at NV-a (a supposed theoretical normal value). However, all three antigens show crossreactivity with tuberculosis patients at different levels. At NV-c (a supposed practical normal value, PNV), this crossreaction significantly decreased in the WML ELISA (PNV = 0.28) and the PGL-I ELISA (PNV = 0.16), and disappeared in the ND-O-BSA ELISA (PNV = 0.20). Under the same conditions, the positivity rates did not decrease significantly in leprosy patients, especially in multibacillary patients. Therefore, we suggest that the PGL-I ELISA in combination with the ND-O-BSA ELISA may be very useful for clinical applications, serodiagnosis, and for the study of subclinical infection in leprosy.

RESUMEN

Se aplico la técnica del inmunoensayo enzimático (ELISA) con el disacárido natural-octil-albúmina sérica bovina (DN-O-ASB) en los sueros de 151 pacientes con lepra, de 20 pacientes con tuberculosis, y de 42 personas sanas de un área no endémica. Los tres ELISAs, con el *Mycobacterium leprae* integral (MLI), con el glicolípido fenólico-1 (GLF-1), y con el DN-O-ASB, son todos altamente sensibles en la detección de anticuerpos contra el *M. leprae.* Los resultados indican que la actividad serológica muestra correlaciones positivas altamente significativas entre los 3 tipos de antígenos usados. El grado de correlación positiva fue del 100% con el GLF-1, y del 97.4% con el MLI y con el DN-O-ASB en los pacientes con lepra, y del 0% con

cualquier antígeno usado en personas normales a un supuesto valor teórico normal (VN-a). Sin embargo, los 3 antígenos muestran reactividad cruzada con pacientes tuberculosos a diferentes niveles. A un supuesto valor práctico normal, VPN (NV-c), esta reactividad cruzada disminuyó significativamente en el ELISA con MLI (VPN = 0.28) y en el ELISA con GLF-1 (VPN = 0.16), y desapareció en el ELISA con el DN-O-ASB (VPN = 0.20). Bajo las mismas condiciones, el grado de positividad no disminuyó significativamente en los pacientes con lepra, especialmente en los multibacilares. Por lo tanto, sugerimos que el ELISA con el GLF-1 en combinación con el ELISA con DN-O-ASB, podría ser muy útil en el serodiagnóstico clínico de la enfermedad y en el estudio de la infección subclínica en la lepra.

RÉSUMÉ

On a étudié au moyen d'une épreuve ELISA une albumine naturelle du serum de boeuf, disaccharide-octyl (ND-O-BSA), dans des échantillons de serum provenant de 151 malades de la lépre, de 20 sujets atteints de tuberculose, et de 42 individus normaux d'une zone non-endémique. Les trois épreuves ELISA, l'une menée avec *Mycobacterium leprae* (WML), la seconde avec l'antigène phenolglycolipidique-I (PGL-I), et ND-O-BSA, étaient très sensibles pour détecter des anticorps contre *M. leprae.* Ces résultats indiquent que l'activité sérologique présentait des corrélations positives et hautement significatives entre les trois types d'antingènes utilisés. Les taux de positivité étaient de 100% avec le PGL-I et de 97, 4% avec WML et ND-O-BSA chez des personnes normales au NV-a, une valeur supposée comme normale sur des bases théoriques.

Néanmoins, les trois antigènes montraient une réactivité croisée, à différents niveaux, chez des malades atteints de tuberculose. Aux valeurs supposées pratiquement normales (PNV), cette réactivité croisée diminuait significativement avec l'ELISA WML (PNV = 0, 28) et l'ELISA PGL -I (PNV = 0,016), et disparaissait pour l'ELISA ND-O-BSA PNV (= 0, 20). Dans les mêmes conditions, les taux de positivité ne diminuaient pas significativement chez les malades atteints de lèpre multibacillaire. Dès lors, on suggère que l'ELISA PGL-I en combinaison avec l'ELISA ND-O-BSA, peut être fort utile pour des applications cliniques, pour le diagnostic sérologique, et pour l'étude de l'infection infraclinique dans la lèpre.

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