ELISA Detection of IgM Antibodies Against Phenolic Glycolipid-I in the Management of Leprosy: A Comparison Between Laboratories[1]

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Phenolic glycolipid-I (PG), a phthiocerol-containing surface glycolipid of *Mycobacterium leprae,* offers significant potential in the serodiagnosis of leprosy ([2]). In addition to detecting new cases of leprosy, the quantitative decrease of anti-PG IgM with treatment of disease ([3, 4]), and the correlation of anti-PG IgM with the bacterial index (BI) ([8]) offers additional potential for monitoring disease activity in patients under therapy. Several laboratories are actively engaged in research on the serodiagnosis of leprosy using PG in ELISA, with individual laboratory variations in ELISA technology, including employing different methods of coating the water-insoluble PG onto microtiter plates. The Seattle laboratory employs deacylated PG, which is more water miscible than native PG ([16]). The Colorado laboratory sonicates native PG into an aqueous suspension ([4]). The New York laboratory, as seen in the present work, incorporates PG into liposomes with other lipids.

Because of the potential for utilizing PG antibodies for serodiagnostic screening and for therapeutic monitoring, we conducted an external comparison between laboratories prior to clinical development.

MATERIALS AND METHODS

The determination of anti-PG IgM levels was done by different ELISA techniques in Colorado, Seattle, and New York on sera from leprosy patients, contacts and controls from the New York metropolitan area. Frozen serum aliquots were sent to the Colorado and Seattle laboratories. The aliquots were coded so that antibody determinations were done blind. Leprosy patients were clinically and histologically classified according to the Ridley-Jopling scale ([11]). The bacterial index (BI) was measured on a semilogarithmic scale (0–6+) approximating that of Ridley ([10]). Histology and BI were determined on punch biopsies at the National Hansen's Disease Center, Carville, Louisiana, U.S.A., by two pathologists (C. K. Job, J. A. Freeman), whose agreement on BI was good ($r = 0.94$). Contacts of leprosy patients consisted of household contacts (HC) and nosocomial contacts (NC). Healthy subjects were used as normal controls (Cont).

New York laboratory. ELISA screening of sera for PG-antibodies was performed with PG incorporated into liposomes, as described for antibodies against glycosphingolipids ([12]). PG-liposomes were prepared as described by other authors ([3]). PG (armadillo-derived phenolic glycolipid-I, kindly provided by P. J. Brennan under NIH contract), sphingomyelin, cholesterol, and dicetyl phosphate (0.1:2.0:1.5:0.2 molar ratio) were combined in chloroform/methanol (2:1 v/v). The solvent was removed by rotary evaporation, and the lipid mixture was sonicated (Branson Sonifier) in Tris-

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buffered saline (0.15 M NaCl, 20 mM Tris, pH 8.0) for 3 min (300 μg PG/ml). Control liposomes without PG were prepared as above. Flat-bottomed, polystyrene microtiter plates (Dynatech, Alexandria, Virginia, U.S.A.) were coated by incubation with 100 μl of the PG-liposome suspension (diluted to 2.5 μg PG/ml in Tris-buffered saline), or with the control-liposome suspension, for 18 hr at 37°C. After washing three times with phosphate buffered saline (PBS) (0.1 M phosphate buffer, pH 7.2, 0.15 M NaCl), the plates were incubated for 1.5 hr at 37°C with 200 μl of 3% (w/v) bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) in PBS (BSA-PBS). BSA-PBS was replaced by 100 μl of test serum in duplicate diluted 1:20 in BSA-PBS. A positive lepromatous serum pool was included as reference on each plate. After incubation with test serum for 1.5 hr at 37°C, the plates were again washed three times with PBS and incubated for 1.5 hr at 37°C with 100 μl of peroxidase-conjugated goat anti-human IgM (Cappel Laboratories, Westchester, Pennsylvania, U.S.A.), diluted 1:1000 in BSA-PBS. After washing three times with PBS, 100 μl of substrate solution, containing 1.8 mM 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Boehringer, Indianapolis, Indiana, U.S.A.) and 0.1 mM H_2O_2 in 0.1 M phosphate buffer, pH 7.0, were added for 1 hr at room temperature. The reaction was stopped with 100 μl of 0.32% (w/v) NaF and the extinction (E) was read at 405 nm using a Titertek microtiter plate reader. The results were expressed as $\Delta E = E$ (PG-liposome coat) $- E$ (control liposome coat). Based on preliminary experiments with control sera, a value of $\Delta E \geq 0.10$ has been chosen as a positive value for anti-PG IgM. This figure is greater than the mean plus three standard deviations (S.D.) for 35 control sera tested (0.085). Out of 36 control sera tested only one, a consistently false-positive, has exceeded this value.

In addition, an ELISA was performed using an *o*-phenylenediamine (OPD) substrate solution. The OPD ELISA procedure was identical to the ABTS ELISA with the following exceptions: a) Sera were diluted 1:300 in BSA-PBS. b) The substrate solution contained 2.2 mM OPD (Sigma) and 4 mM H_2O_2 in citrate-phosphate buffer pH 5.0. c) The reaction was stopped after 30 min with 100 μl/well 2.5 N H_2SO_4 and absorbance read at 492 nm. Using the same criteria for seropositivity as the ABTS assay, a $\Delta E \geq 0.20$ was considered positive for OPD.

Colorado laboratory. PG was suspended in ammonium acetate buffer pH 8.2 (100 μg PG/ml) by direct sonication. The suspension was diluted to 2 μg/ml with the same buffer. U-bottomed polystyrene microtiter plates (Dynatech) were coated with 50 μl/well of the PG suspensions. The plates were incubated at 37°C for 14 to 16 hr in a moist chamber. After washing with PBS, the plates were incubated with 100 μl/well of 5% BSA-PBS for 1 hr at 37°C in the moist chamber. BSA-PBS was replaced with 50 μl of test serum diluted 1:300 in PBS containing 20% normal goat serum (PBS-NGS) or 5% BSA-PBS. The plates were incubated at 37°C for 1 hr and then washed four times with PBS. Goat anti-human IgM-peroxidase (Cappel), diluted 1:1000 in PBS-NGS or 5% BSA-PBS, was added to the plates and incubated for 1 hr. Following incubation with conjugate, the plates were washed five times with PBS, and 50 μl/well of the substrate solution (2.2 mM OPD + 5 mM H_2O_2 in citrate-phosphate buffer) was added. The plates were incubated for 30 min at 37°C, the reaction stopped with 2.5 N H_2SO_4, and the absorbance read at 488 nM (A_{488}). Based on previous studies ([4]), $A_{488} \geq 0.12$ was considered positive, representing the mean of 169 control sera plus three standard deviations.

Seattle laboratory. PG was deacylated and recovered as previously described ([16]). The deacylated PG was vortexed with distilled water (50 μg/ml), incubated at 55°C for 15 min, then diluted to 5 μg/ml. Polystyrene microtiter plates (Linbro, Hamden, Connecticut, U.S.A.) were coated with 0.1 ml/well of the PG suspension on one half of the plate and 0.1 ml/well distilled water on the other half. The plates were incubated overnight at 37°C, washed four times with PBS, and then incubated with 0.1 ml/well 5% BSA-PBS for 2 hr at 37°C. BSA-PBS was replaced with 0.1 ml duplicates of serum diluted 1:20 in PBS + 5% fetal calf serum (FCS), and the plates were incubated

TABLE 1. *Mean absorbance levels (± S.D.) and number of positive/negative sera by Ridley-Jopling class for ELISA techniques.*

	New York laboratory		Colorado laboratory	Seattle laboratory
	ABTS	OPD		
	Absorbance (+/−)	Absorbance (+/−)	Absorbance (+/−)	Absorbance (+/−)
LL	0.30 ± 0.32 (71/42)	0.33 ± 0.28 (51/34)	0.36 ± 0.46 (59/42)	0.37 ± 0.29 (50/14)
BL	0.47 ± 0.34 (27/6)	0.46 ± 0.24 (15/1)	0.61 ± 0.56 (22/6)	0.54 ± 0.32 (11/1)
BB	0.25 ± 0.21 (4/2)	0.53 ± 0.41 (3/1)	0.22 ± 0.21 (3/3)	0.32 ± 0.22 (2/1)
BT	0.11 ± 0.21 (11/24)	0.22 ± 0.18 (9/18)	0.11 ± 0.21 (5/27)	0.18 ± 0.22 (10/6)
TT	0.02 ± 0.03 (0/4)	0.20 ± 0.08 (2/2)	0.02 ± 0.01 (0/4)	NA[a]
NC[b]	0 ± 0.02 (0/14)	0.09 ± 0.05 (0/14)	0.06 ± 0.03 (0/3)	0.03 ± 0.06 (1/12)
HC[c]	0.03 ± 0.09 (1/15)	0.13 ± 0.11 (2/14)	0.17 ± 0.18 (1/1)	0.01 ± 0.03 (0/14)
Cont[d]	0.07 ± 0.17 (2/11)	0.06 ± 0.13 (1/10)	NA	0.06 ± 0.11 (2/11)

[a] NA = none assayed.
[b] NC = nosocomial contacts.
[c] HC = household contacts.
[d] Cont = controls.

for 2 hr at 37°C. The plates were then washed four times with PBS, and goat anti-human IgM-peroxidase (Cappel) diluted 1:1000 in PBS + 1% FCS was added. The plates were then incubated for 1 hr at 37°C, washed four times with PBS, and the substrate solution (0.4 mM OPD, 1 mM H_2O_2 in PBS + 0.05% Tween 20, 0.1 ml/well) was added. The plates were incubated at room temperature for 20 min, the reaction stopped by the addition of 25 μl/well of 8 N H_2SO_4, and read at 492 nm. Results were expressed as: $\Delta A_{492} = A_{492}$ PG coated wells $- A_{492}$ uncoated wells. $\Delta A_{492} \geq 0.10$ was considered positive, based on preliminary studies ([15]) which indicated the ΔA_{492} range of normal human sera to be -0.05 to 0.05.

Statistical analysis. Statistical analysis was done by computer using BMDP statistical packages ([6]). Polynomial regression analysis was performed on the absorbance values obtained by the different ELISA techniques on multibacillary (LL, BL) and paucibacillary (BB, BT, TT) leprosy patients. Using anti-PG IgM values of leprosy patients' sera multiple correlations of IgM and erythema nodosum leprosum (ENL) were done with the BI as an independent variable for each technique to determine the effect of ENL on the relationship of the BI and anti-PG IgM.

RESULTS

The mean absorbance values and the number of positive/negative sera for each ELISA technique by Ridley-Jopling classification are presented in Table 1.

Agreement on seropositivity/negativity between the ELISA techniques for the leprosy patients' sera ranged from 61.7% (New York OPD: Seattle lab) to 85.4% (ABTS: Colorado lab) (Table 2). In cases of disagreement, the Seattle ELISA appeared to

TABLE 2. *Agreement and disagreement of seropositivity/negativity between ELISA techniques.*

ELISA technique	Leprosy patients					Contacts and controls				
	++[a]	−−[b]	+−[c]	−+[d]	%[e]	++	−−	+−	−+	%
New York ABTS: New York OPD	62	40	17	17	75.0	2	37	0	1	97.5
New York ABTS: Colorado	83	63	18	7	85.4	1	3	0	0	100
New York ABTS: Seattle	56	18	4	17	77.9	2	37	0	1	97.5
New York OPD: Colorado	54	47	19	8	78.9	1	3	0	0	100
New York OPD: Seattle	26	11	1	22	61.7	1	35	1	1	94.7
Colorado: Seattle	39	17	1	18	74.7	0	2	0	0	100

[a] Positive by both assays.
[b] Negative by both assays.
[c] Positive by first assay, negative by second assay.
[d] Negative by first assay, positive by second assay.
[e] Percent agreement.

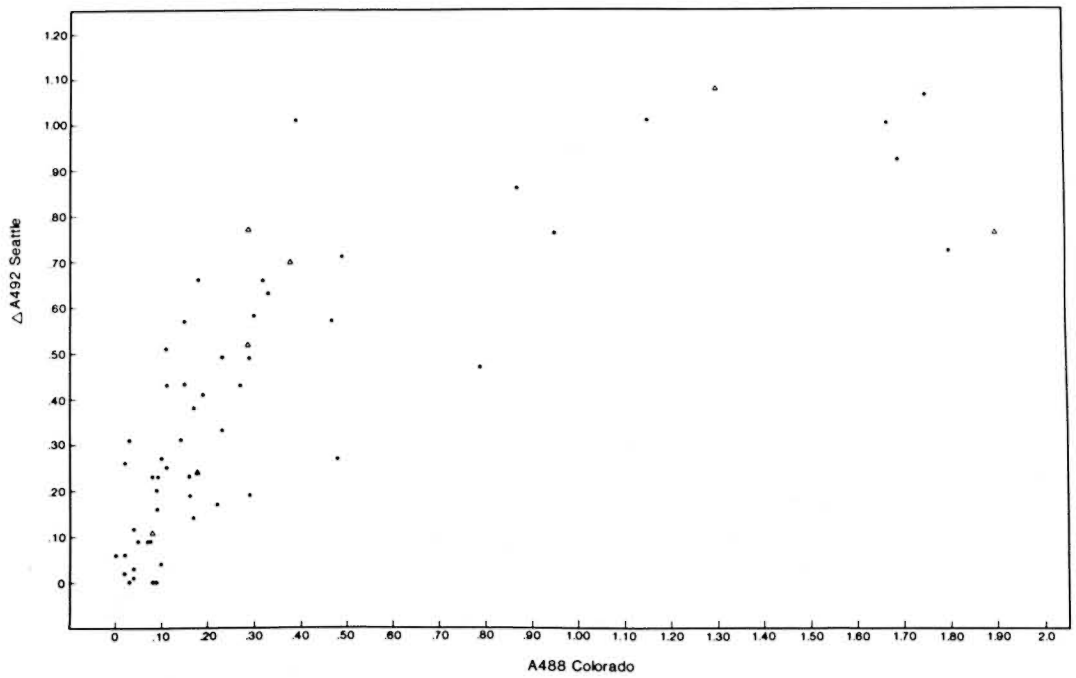

FIG. 1. Correlation between absorbance values for anti-PG IgM obtained on multibacillary leprosy (LL and BL) sera by Seattle and Colorado ELISAs. ● = LL; △ = BL.

detect leprosy patients' sera as positive more often than the other techniques, and the New York lab's ABTS and the New York OPD methods appeared to detect slightly more leprosy sera as positive than the Colorado ELISA. For the most part, these cases of disagreement involved sera that were weakly positive by one assay. For example, the mean absorbance levels (±S.D.) for leprosy sera detected by the Seattle assay that other assays called negative were 0.22 ± 0.11 vs 0.04 ± 0.03, 0.23 ± 0.10 vs 0.13 ± 0.14, and 0.23 ± 0.13 vs 0.07 ± 0.03 for Seattle vs New York ABTS, New York OPD, and Colorado respectively. Agreement on positivity/negativity for patient contacts and the controls was very good. The New York OPD assay detected as positive one HC that the New York ABTS and Seattle ELISA called negative ($\Delta E = 0.24$ vs $\Delta E = -0.04$ and $\Delta A_{492} = 0.01$, respectively, not assayed by Colorado lab), while the Seattle ELISA detected as positive one NC that was negative by the New York ABTS and New York OPD ($\Delta A_{492} = 0.21$ vs $\Delta E = 0.02$ and $\Delta E = 0.09$, respectively; not assayed by Colorado

TABLE 3. *Correlation between ELISA techniques for multibacillary and paucibacillary leprosy patients.*

ELISA technique	Multibacillary[a]			Paucibacillary[b]		
	No.	r	Form of regression line	No.	r	Form of regression line
New York ABTS: New York OPD	101	0.7428	Linear	35	0.9311	Nonlinear
New York ABTS: Colorado	129	0.9201	Nonlinear	42	0.9778	Linear
New York ABTS: Seattle	76	0.8077	Linear	19	0.6594	Linear
New York OPD: Colorado	98	0.7769	Nonlinear	34	0.9264	Nonlinear
New York OPD: Seattle	48	0.7485	Nonlinear	12	0.9229	Nonlinear
Colorado: Seattle	59	0.8536	Nonlinear	16	0.8394	Linear

[a] Multibacillary = LL, BL.
[b] Paucibacillary = BB, BT, TT.

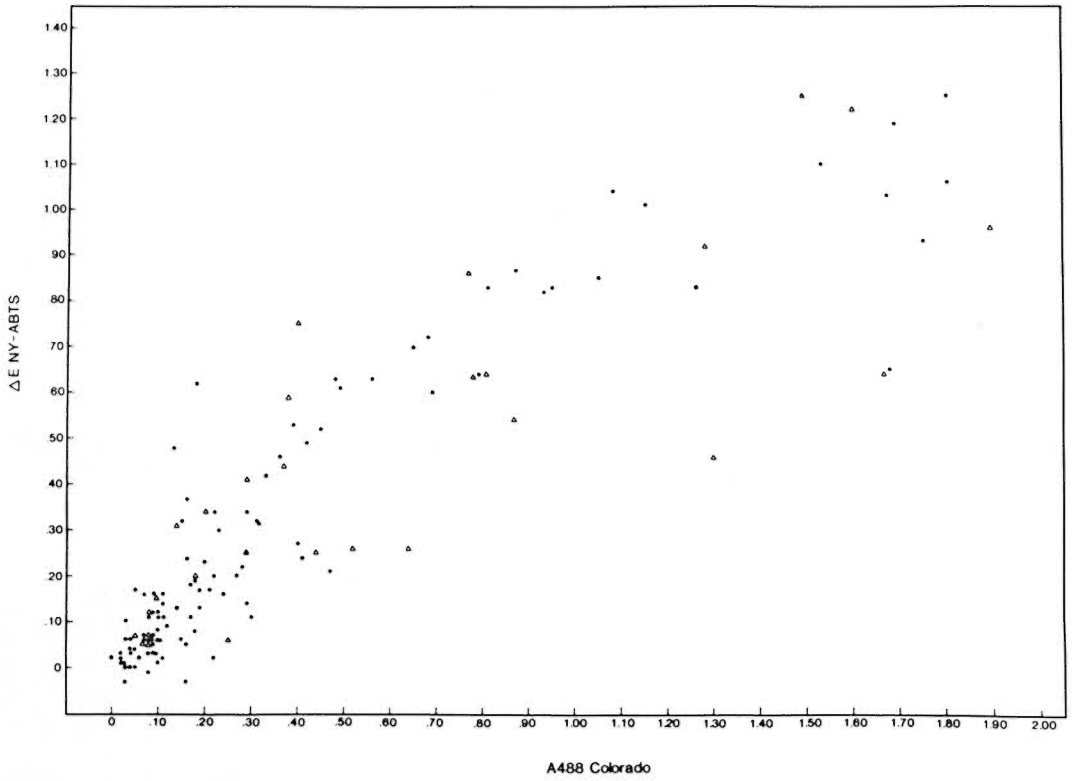

FIG. 2. Correlation between absorbance values for anti-PG IgM obtained on multibacillary leprosy sera by New York ABTS and Colorado ELISAs. ● = LL; △ = BL.

lab). One HC (a contact of BL patient) was found to be positive by the New York ABTS, New York OPD, and Colorado ELISA ($\Delta E = 0.36$, $\Delta E = 0.46$, $A_{488} = 0.30$, respectively; not assayed by Seattle lab). The first serum of one healthy control was found positive by the New York ABTS, New York OPD, and Seattle ELISA ($\Delta E = 0.48$, $\Delta E = 0.40$, $\Delta A_{492} = 0.30$, respectively; sample not assayed by Colorado lab). The second serum from this subject, taken 3 months later, tested positive by the New York ABTS and Seattle ELISA ($\Delta E = 0.41$ and $\Delta A_{492} = 0.32$, respectively; not assayed the New York OPD or Colorado lab).

The correlation between techniques was generally good for both multibacillary (LL and BL) and paucibacillary (BB, BT, TT) leprosy patients (Table 3). Figures 1 and 2 depict data for two nonlinear relationships (Seattle vs Colorado-multibacillary and New York ABTS vs Colorado-multibacillary), while Figures 3 and 4 show linear relationships (New York ABTS vs Seattle-multibacillary and New York ABTS vs Colorado-paucibacillary). It may be seen that among patients with very high levels of antibody the Colorado assay tends to give the highest absorbance readings (Figs. 1 and 2).

The New York laboratory has reported a positive linear correlation between the BI and anti-PG IgM, and that ENL significantly lowers this relationship ([8, 9]). The ENL effect was seen with ABTS but not with OPD. Data from each ELISA technique were analyzed to see if these relationships were detected. All four ELISA techniques detected a significant rise in anti-PG IgM level with an increasing BI (Table 4). ENL significantly lowered the relationship between anti-PG IgM and the BI when data from the New York ABTS and the Colorado assay were used (Table 5). Using data from the New York OPD and the Seattle lab, the regression lines for the BI vs the anti-PG IgM for non-ENL patients had greater slopes than

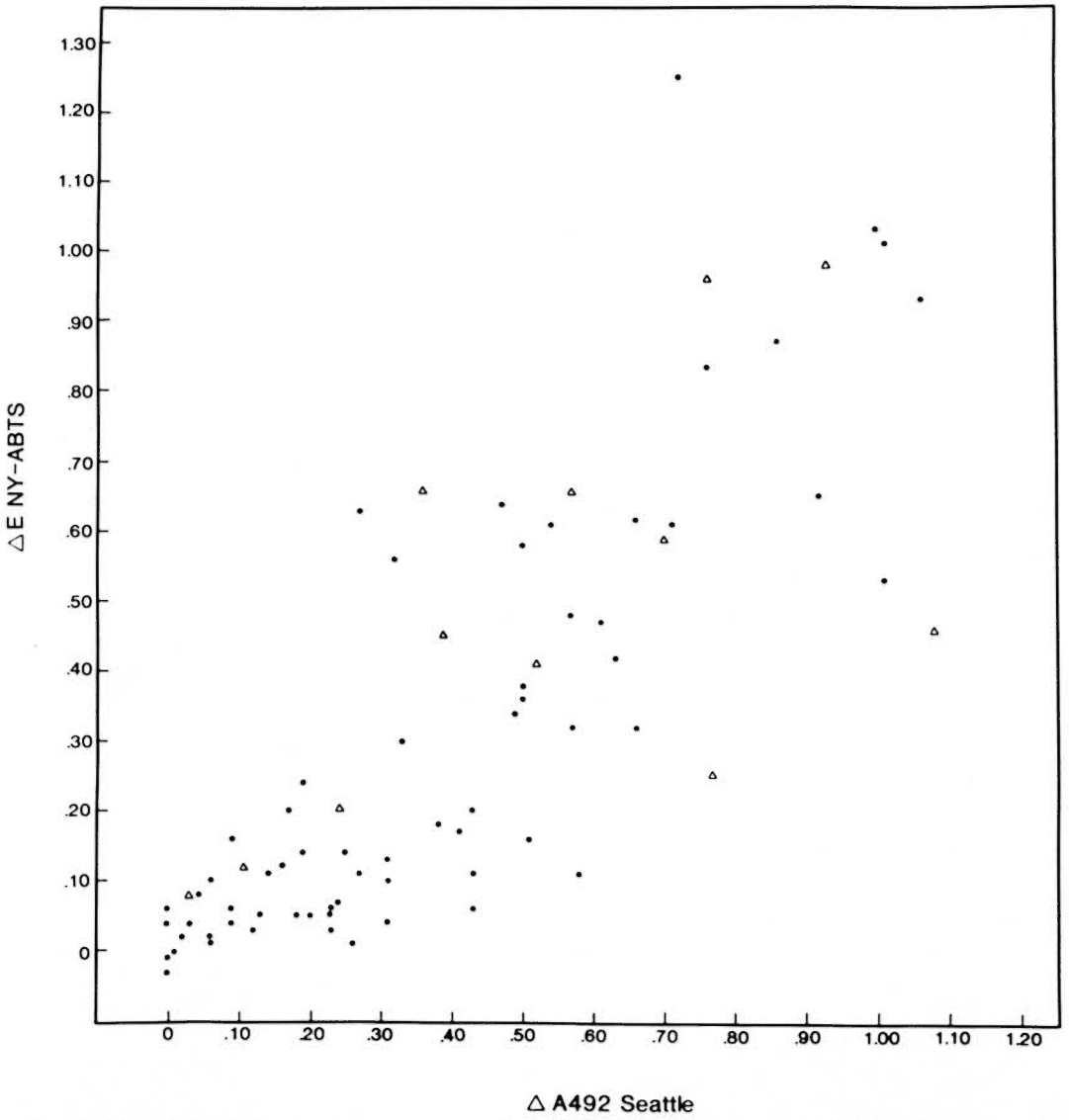

FIG. 3. Correlation between absorbance values for anti-Pg IgM obtained on multibacillary leprosy sera by New York ABTS and Seattle ELISAs. ● = LL; △ = BL.

TABLE 4. *Relationship between BI and anti-PG IgM as detected by four ELISA techniques.*

ELISA technique	No.	Regression lines	r[a]
New York ABTS	191	IgM = 0.0798(BI) + 0.1674[b]	0.4957
New York OPD	136	IgM = 0.0711(BI) + 0.2033[b]	0.4304
Colorado	171	IgM = 0.1189(BI) + 0.1658[b]	0.5245
Seattle	95	IgM = 0.0591(BI) + 0.2748[b]	0.3900

[a] Correlation coefficients for BI and anti-PG IgM.
[b] Relationship between BI and anti-PG IgM significant at $p < 0.01$.

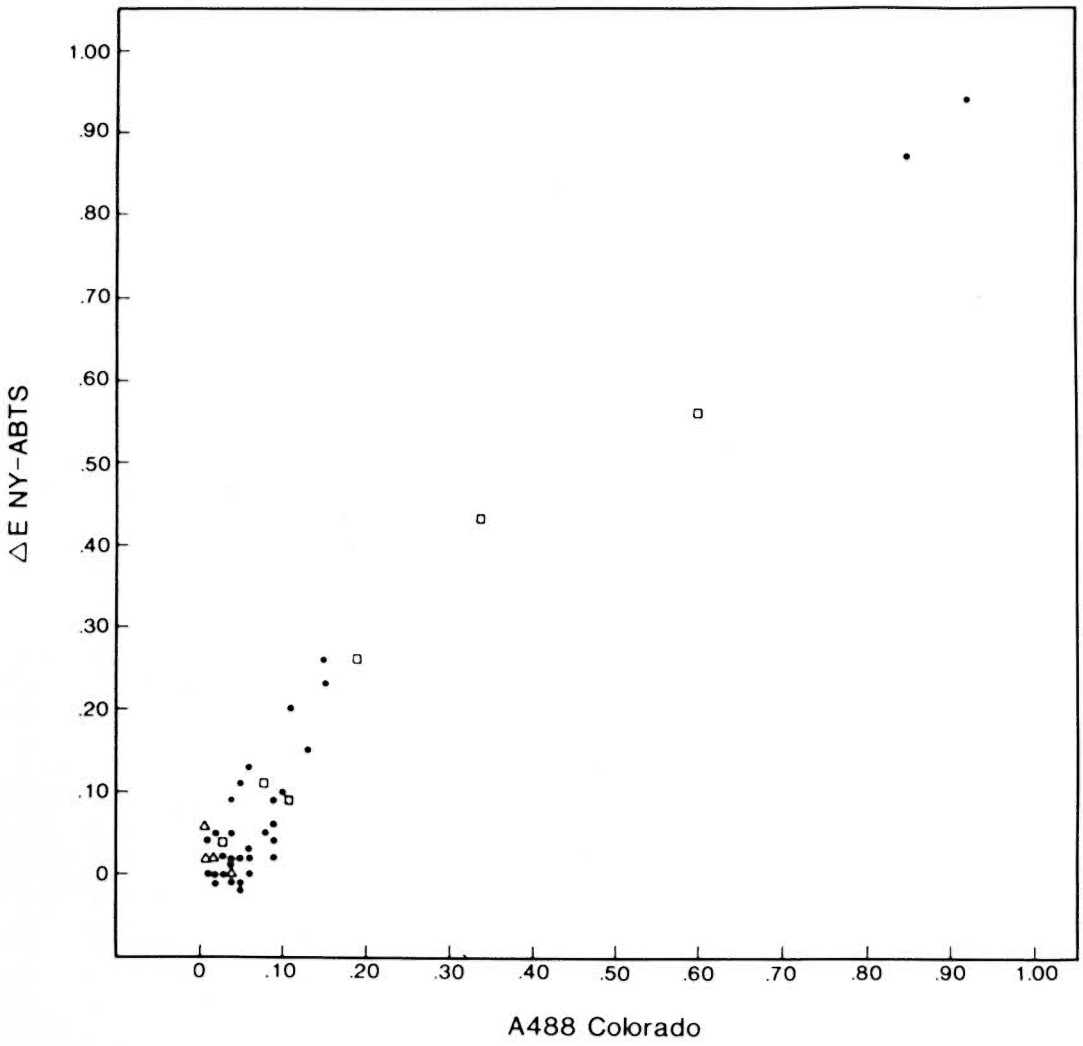

FIG. 4. Correlation between absorbance values for anti-PG IgM obtained on paucibacillary (BB, BT, TT) leprosy sera by New York ABTS and Colorado ELISAs. □ = BB; ● = BT; △ = TT.

those for ENL patients. However, the difference in the lines was not enough to achieve statistical significance (Table 5).

DISCUSSION

It can be concluded that despite differences in technique, excellent correlation was achieved between the New York, Colorado, and Seattle laboratories in the detection of anti-PG IgM. There are several differences in the methodology of these ELISA techniques that could have a bearing on the discrepancies noted among the assays. The method of coating and the amount of PG used in each well are possible sources of variation. Differences in exposure of antigenic determinants may occur as a result of coating technique. The Colorado assay uses the least PG per well (0.1 μg), the New York ABTS and New York OPD use 0.25 μg/well, while the Seattle ELISA uses 0.5 μg/well of deacylated PG (antigenically equivalent to approximately 1 μg/well of native glycolipid). This may explain why the Seattle assay detected low-titer leprosy patients missed by the other assays and why the New York ABTS and New York OPD assays appeared to detect more low-titer patients than did the Colorado lab. Another difference in the ELISA methodology among these assays

TABLE 5. *Effect of ENL on relationship between BI and anti-PG IgM measured by four different ELISA techniques.*

ELISA technique	Regression lines			
	No.	ENL	No.	Non-ENL
New York ABTS	76	IgM = 0.0499(BI) + 0.1355[a]	115	IgM = 0.1239(BI) + 0.1847[a]
New York OPD	49	IgM = 0.0409(BI) + 0.2418	87	IgM = 0.0962(BI) + 0.1934
Colorado	68	IgM = 0.0771(BI) + 0.1397[a]	103	IgM = 0.1737(BI) + 0.1833[a]
Seattle	37	IgM = 0.0382(BI) + 0.2525	58	IgM = 0.0913(BI) + 0.2857

[a] ENL and non-ENL regression lines significantly different ($p < 0.01$).

is in the make up of the substrate solutions and the length of time these solutions are allowed to react. Two chromogenic dyes have been used (ABTS in the New York ABTS, OPD in the other ELISAs). The Colorado and the New York OPD assays have 40-fold more H_2O_2 than the New York ABTS, and fourfold more than the Seattle assay. The Seattle ELISA uses about 20% of the concentration of chromogenic dye compared to the other assays. The pH of the substrate buffer (7.0 for the New York ABTS, 5.0 for the other ELISAs) can also affect the rate of reaction for horseradish peroxidase ([14]). Incubation times range from 20 min (Seattle) to 1 hr (New York ABTS). Finally, the different serum concentration used in the assays is another possible source of variability. The New York ABTS and the Seattle assay dilute serum 1:20, while the Colorado and New York OPD assays use a 1:300 dilution.

The detection of a false-positive healthy control is consistent with previous reports of false-positives in nonendemic areas ([4]). The false-positive serum donor, in this case, was an American-born, New York City resident with no known contact with leprosy or other mycobacterial infections.

All four ELISA techniques detected a statistically significant correlation between the BI and anti-PG IgM. The correlation coefficients (ranging from 0.3900 to 0.5245) indicate that factors other than the BI contribute to the level of anti-PG IgM. Slow elimination of the water insoluble PG from the tissues may lead to antigenic persistence. It may also be that genetic factors play a role in response to PG ([13]).

The effect of ENL on the relationship of the BI and anti-PG IgM was significant with the New York ABTS and the Colorado assays but not with the New York OPD and Seattle assays, in which cases the difference between ENL and non-ENL regression lines narrowly failed to reach statistical significance. It should be noted that decreases in anti-PG IgM during ENL episodes have been seen in serial sera ([1]).

Since these three laboratories show external correlation, we have a quality control that will allow for clinical deployment at this time. Reports have indicated that most antibody binding to PG is directed at the terminal 3,6-di-*O*-methyl-β-D-glucopyranose residue of the PG trisaccharide ([4, 7, 17]). The Colorado laboratory has reported on the use of a synthetic glycoconjugate utilizing the terminal two sugar residues of PG covalently attached to BSA in the ELISA, and this has been shown to correlate well with their native PG assay ([4]). Since supplies of armadillo-derived materials are limited, the use of a synthetic glycoconjugate may be an important tool for widespread clinical application.

SUMMARY

IgM antibodies to the phenolic glycolipid-I (PGL-I) antigen of *Mycobacterium leprae* were detected by different ELISA techniques in three laboratories (in New York, Colorado, Seattle, U.S.A.). The agreement on seropositivity and overall correlation between techniques was excellent. A positive linear correlation between the bacterial index (BI) and anti-PGL-I IgM, previously reported by the New York laboratory, was detected by all techniques. The role of erythema nodosum leprosum in decreasing the relationship of BI versus anti-PGL-I IgM was seen by the New York laboratory with sera diluted 1:20 and ABTS substrate solution and by the Colorado laboratory but not by New York with sera at

1:300 and OPD substrate or by the Seattle laboratory.

RESUMEN

En tres laboratorios diferentes (Nueva York, Colorado y Seattle, E.U.A.) y con técnicas de ELISA particulares, se investigó la presencia de anticuerpos IgM contra el glicolípido fenólico I (GLP-I) del *Mycobacterium leprae*. La correlación entre las diferentes técnicas resultó excelente. Con todas las técnicas se encontró una correlación lineal positiva entre el índice bacteriano (IB) y la concentración de IgM anti-GLP-I, observación previamente reportada por el laboratorio de Nueva York. El papel del eritema nodoso leproso en la disminución de la relación BI:IgM-anti-GLP-I fue notado por el laboratorio de Nueva York en sueros diluidos 1:20 y usando ABTS en la solución del substrato. También se observó en el laboratorio de Colorado pero no en el de N.Y. ni en el de Seattle, con los sueros diluidos 1:300, usando OPD en la solución del substrato.

RÉSUMÉ

Des anticorps IgM à l'antigène phéno-glycolipidique (PGL-I) de *Mycobacterium leprae* ont été détectés par différentes techniques ELISA dans trois laboratoires aux Etats-Unis (New York, Colorado, Seattle). La concordance des résultats sérologiques positifs dans les techniques, de même que la corrélation totale, étaient excellentes. Une corrélation linéaire positive entre l'index bactériologique (IB) et les IgM anti-PGL-I, a été constatée avec chacune des trois techniques, ce qui confirme les résultats rapportés déjà par le laboratoire de New York. Ce laboratoire, utilisant des échantillons de sérum dilués à 1:20 et une solution de substrat ABTS, avait constaté une diminution de la relation entre l'index bactériologique (IB) et les IgM anti-PGL-I en cas d'érythème noueux lépreux; le laboratoire du Colorado, mais non ceux de New York et ceux de Seattle, avait fait la même constatation avec des échantillons de sérum dilués à 1:300 et avec un substrat OPD.

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