Development of an ELISA for Detection of Antibody in Leprosy[1]

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The development of an effective immunoassay to detect antibody in preclinical leprosy would greatly aid the effectiveness of leprosy control programs. Such an assay could both facilitate early diagnosis of the disease and could decrease incidence of the disease by early detection of multibacillary individuals.

Several laboratories have developed immunoassays for leprosy. These assays include: the fluorescent leprosy antibody absorption test (FLA-ABS) developed by Abe, et al. ([1]), the radioimmunoassay (RIA) with antigen 7 developed by Harboe, et al. ([6]), and the enzyme-linked immunosorbent assay (ELISA) with arabinomannan developed by Miller and Buchanan (personal communication). In contrast to the immunoassays which use extracts of mycobacteria as the antigen, we have chosen to use whole cells. Our choice is based on the premise that cell surface antigens should be easily recognized by the immune system of the host. It is also possible that antigens which have been extracted from cells may either have altered determinants or have been denatured. Another possibility is that extracted antigens may not be presented in immunoassays in the same way they are presented on cells. In addition to the application of whole cells to the ELISA, we have chosen not to absorb sera prior to testing, but to establish a background for "natural antibody" levels to common determinants of mycobacteria. In the results reported here, we have surveyed several related organisms in the corynebacteria/mycobacteria groups, including *Mycobacterium leprae*, to determine the best candidate for a whole cell antigen ([4]). We have also developed methods for coating whole bacteria to microtiter plates and a means to compensate for natural antibody levels to mycobacteria found in most individuals.

MATERIALS AND METHODS

Sera. Sera from biopsy-classified lepromatous, tuberculoid, and borderline patients as well as negative controls were generously provided by Dr. E. Reichert and the Hansen's Disease Control Program at Leahi Hospital, Honolulu, Hawaii, and Dr. R. Worth, University of Hawaii School of Public Health. The negative control sera were collected from individuals who had not lived in leprosy endemic areas and were tuberculin negative. Seven lepromatous and seven negative control sera were pooled to create positive and negative controls for the ELISA. Each serum was tested in duplicate wells. The optical density (OD) was read at 492 nm by Titertek Multiskan (Flow Laboratories, McLean, Virginia, U.S.A.). The data are reported as averaged duplicate wells.

Cultures. Mycobacteria were cultivated at 37°C on Löwenstein-Jensen medium and incubated for 4–18 days depending on growth rate. Corynebacteria were cultivated at 37°C for 48 hr on Trypticase Soy blood agar plates.

Preparation of ELISA plates. Two methods of coating whole cells were compared. The cells used as ELISA antigens, *M. smegmatis* (TMC 1515), *M. vaccae*, *M. scrofulaceum*, *Corynebacterium diphtheriae*, and *C. xerosis*, were suspended in coating buffers at a concentration of 0.15 absorbence units at OD 420 nm. The two pH 8.2 buffers used were 0.15 M Na borate and a volatile 0.01 M ammonium acetate carbonate buffer. The cells were then coated onto wells of flat-bottom microtiter plates (Linbro, Cat. No. 76-381-04; Flow Laboratories) in a volume of 200 µl per well. Borate-coated plates were incubated overnight at 4°C; while carbonate coated plates were dried for 48 hr at 37°C.

The coating concentration of *M. leprae* was established by adjusting the number of

organisms isolated from armadillo tissue in the volatile acetate carbonate buffer to 0.07 OD units at 420 nm (approximately 2 × 10^7/ml). The suspension was also dried onto microtiter plates. The _M. leprae_ lot was provided under a National Institutes of Health (NIH) contract by Dr. P. Brennan, Colorado State University, Fort Collins, Colorado, U.S.A., and was labeled "liver #212 3-12-81."

Autoclaved whole-cell antigens were obtained by autoclaving the organisms in borate buffer for 30 min at 121°C. After centrifugation, the cells were resuspended in the ammonium acetate carbonate buffer to specified OD at 420 nm.

ELISA. Two hundred μl of serum diluted 1/200 in phosphate buffered saline containing 0.1% Tween, pH 7.2 (PBST) was added to duplicate wells and incubated for 2 hr at 25°C. After four washes with PBST, horseradish peroxidase enzyme (Cappel Lab, Inc., Cochranville, Pennsylvania, U.S.A.) conjugated to goat IgG reacting with human IgGAM or IgG (200 μl of 1:8000 in PBST) was added and incubated 30 min at 37°C. Plates were washed four times and 200 μl of substrate (0.003% hydrogen peroxide) and color reagent (0.01% o-phenylenediamine) in PBST or citrate buffer pH 5 (24.3 ml of 0.1 M citric acid, 25.7 ml of 0.2 M Na_2HPO_4, and 50 ml of H_2O) were added and incubated in the dark for 30 min at 37°C ([2]). The reaction was stopped by the addition of 25 μl of 8 N sulfuric acid and read at 492 nm. The test results are reported as the average of duplicate wells. If the difference in the OD values of the duplicate wells exceeded 10% of the mean, the test was repeated. Unless otherwise noted, the reactivity is expressed as OD_{492} of test sera minus OD_{492} of pooled negative sera.

RESULTS

Coating method. The cells coated by the ammonium acetate carbonate buffer method were found to be more reactive than cells coated with borate buffer in detecting antibodies in pooled lepromatous sera (Table 1). A comparison of reactivity to pooled lepromatous sera with _M. smegmatis, C. diphtheriae, C. xerosis, M. scrofulaceum,_ and _M. vaccae_ coated by the two methods was made. As can be seen in Table 1, the

TABLE 1. _Comparison of ELISA antigen coating buffers with the reactivity of pooled lepromatous sera_[a] _to mycobacteria and cornebacteria._

Species	Non-volatile[b]	Volatile[c]	Fold increases of volatile/non-volatile
M. smegmatis[d]	0.312[e]	1.054[e]	3.4
C. diphtheriae	0.592	0.665	1.1
C. xerosis	0.547	0.599	1.1
M. scrofulaceum	0.099	0.158	1.6
M. vaccae	0.042	0.082	1.9

[a] Lepromatous sera represent a pool from seven patients. Control sera represent a pool from seven individuals who were skin test negative for tuberculosis and had not lived in endemic areas for leprosy. Sera pools were diluted 1:200.

[b] 0.15 M Na borate, pH 8.2.

[c] 0.01 M ammonium acetate carbonate, pH 8.2.

[d] The concentration of each antigen in the coating buffers was adjusted to 0.15 OD units at 420 nm.

[e] ELISA OD_{420} readings for each organism were determined by subtracting the OD_{420} values given by pooled negative sera.

carbonate method of coating resulted in a 1.1- to 3.4-fold increase in OD readings when compared to the borate method.

Reactivity of whole-cell antigens. When the reactivity of the mycobacteria and corynebacteria listed in Table 1 was compared, it was found that the carbonate-coated _M. smegmatis_ reacted best with the pooled lepromatous sera. The OD reading for _M. smegmatis_ was 1.054 OD units. The next highest reading was _C. diphtheriae_ at 0.665 OD units, and the least reactive was _M. vaccae_ at 0.082 OD units.

In Table 2 a comparison of the ELISA reactivity against lepromatous sera by autoclaved _M. smegmatis_ (_M. smegmatis-A_) and unautoclaved _M. smegmatis_ was made. It was found that the autoclaving of _M. smegmatis_ resulted in increased reactivity in the ELISA with lepromatous patients. This increase was found to be 1.4- to 3.2-fold (Table 2). It should also be noted that autoclaving the antigen prior to coating resulted in greater separation of reactivity between the lepromatous sera and the negative (control) sera. Serum #50 reacted no higher than the negative pool when the two antigen

TABLE 2. *ELISA reactivity of patient sera to* M. smegmatis *and autoclaved* M. smegmatis (M. smegmatis-*A*).

Sera[a]	M. smegmatis		Ratio[c]
	Unauto-claved[b]	Auto-claved	
002 (LL)	0.28[d]	0.70	2.5
008 (LL)	0.41	1.10	2.7
009 (LL)	1.22	1.73	1.4
083 (LL)	0.22	0.73	3.4
059 (BB/BL)	0.50	0.60	1.2
050 (Inact. LL)	0.30	0.33	1.1
103 (Neg.)	0.17	0.21	1.2
107 (Neg.)	0.37	0.40	1.1
122 (Neg.)	0.28	0.23	0.8

[a] Patient and control (negative) sera were diluted 1:200.

[b] Antigen was coated with the volatile carbonate/acetate buffer at a concentration of 0.15 OD at 420 nm.

[c] Ratio equals fold increase in ELISA values determined by dividing autoclaved units by unautoclaved units.

[d] ELISA OD units read at 492 nm.

TABLE 3. *Titration of lepromatous and negative sera with* M. smegmatis-*A as the* ELISA *antigen.*[a]

Dilution of sera	LL pool[b]	Negative pool[c]
1:200	1.572[d]	0.330
1:400	1.336	0.180
1:800	1.044	0.128
1:1600	0.726	0.044
1:3200	0.548	0.011
1:6400	0.185	ND[e]
1:12,800	0.093	ND
1:25,600	0.037	ND
1:51,200	0.021	ND

[a] The concentration of cells in the volatile coating buffer was adjusted to an OD of 0.15 at 420 nm for the antigen coating.

[b] The LL pool represents pooled sera from seven active lepromatous patients.

[c] The negative pool represents pooled sera from seven individuals who were skin test negative for tuberculosis and had not lived in an endemic area for leprosy.

[d] ELISA OD units read at 492 nm.

[e] ND = not done.

preparations were compared. This patient was an inactive lepromatous leprosy (LL) case with a current Bacterial Index of zero. It was noted that the borderline-borderline lepromatous (BB-BL) patient #59 reacted with an OD of 0.506 units for the unheated *M. smegmatis.* There was no distinction between the BB-BL patient and the LL patients with unheated antigen. When the organisms were autoclaved prior to coating, a distinction could be made with the lowest active LL patient serum reacting with an OD of 0.703 as compared to the OD of 0.595 for the serum from the BB-BL patient.

The results listed in Table 3 compared the serological activity as measured by ELISA of pooled lepromatous sera (LL-pool) and pooled negative (control) sera. Titration of the sera was used to demonstrate relative sensitivity to the assay. An endpoint of 1:12,800 was selected for the LL pool. The titration endpoint for the negative pool was between 1:800 and 1:1200. Based on this endpoint titration data, the sensitivity of the ELISA with *M. smegmatis-A* was approximately ten times greater with lepromatous sera as compared to control sera.

As can be seen in Table 3, it was also found that we could increase sensitivity by diluting test sera to about 1:500. This would result in approximately an eight-fold difference between pooled lepromatous and pooled negative sera.

Patient sera. A total of 114 sera were tested with *M. smegmatis-A* as the antigen. Lepromatous sera were found to be the most reactive, borderline less reactive, and tuberculoid and negative control sera the least reactive. Samples of 51 patient and control sera diluted 1:200 in PBST were tested using peroxidase conjugated to the goat antihuman IgGAM and indicator o-phenylenediamine in PBS buffer (Fig. 1a). OD readings expressed were blanked against a negative serum of OD reading 0.36. The range for lepromatous sera was OD 0.66–1.54 at 492 nm with a mean of 1.04 (denoted by center bar). The 14 borderline patients showed a range of 0–0.47 with a mean of 0.018; 11 tuberculoid sera and 17 negative controls showed ranges of 0–0.16 and 0–0.17, respectively, both having a mean of 0.02.

An additional 63 sera (Fig. 1b) were tested using horseradish peroxidase conjugated to goat antihuman IgG and o-phenylenediamine in citrate buffer, pH 5.0. OD readings were blanked against a pooled negative sera reading of 0.33. The 31 lepromatous sera ranged between 0.346 and 1.449, with a
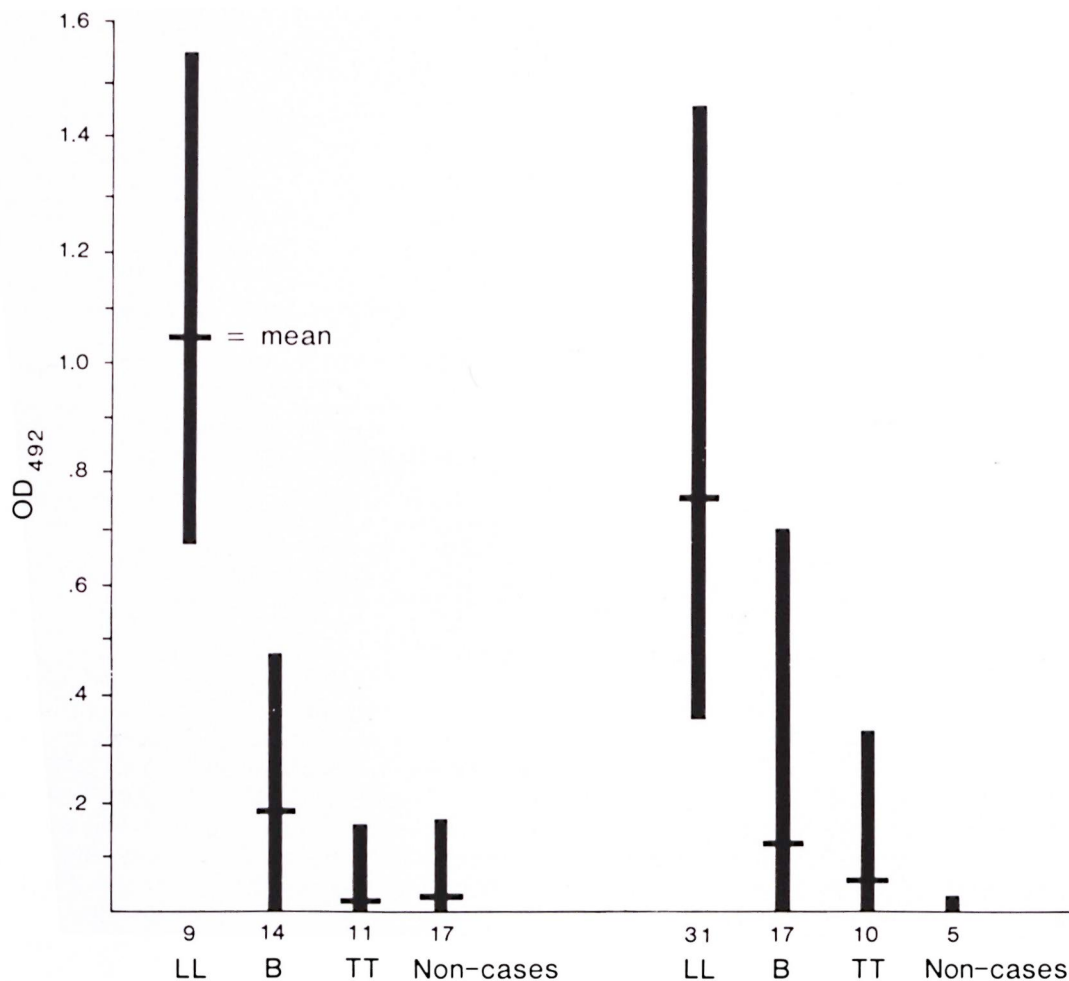
FIG. 1a.   ELISA reactivity of 51 Hawaii sera to *M. smegmatis* antigen (antihuman IgM, IgG, and IgA conjugates with OPD-PBS indicator).

FIG. 1b.   ELISA reactivity of 63 Hawaii sera to *M. smegmatis* antigen (antihuman IgG and OPD citrate indicator).

mean of 0.752; 17 borderline patients showed a range of 0–0.715 with a mean of 0.133; 10 tuberculoid patients had a range of 0–0.334 with a mean of 0.057; 5 negative controls ranged from 0–0.1 with a mean of 0.002.

Comparison of *M. leprae* and *M. smegmatis.* For these comparisons the control or pooled negative sera were not subtracted from the values observed but were expressed as controls in Tables 4 and 5. A comparison of the ELISA reactivity of *M. leprae* and *M. smegmatis* is shown in Table 4. It was found that with the lepromatous pool *M. leprae* was approximately three times more reactive as measured by ELISA

than autoclaved *M. smegmatis*. A similar difference was found with individual LL patient sera. The pooled control sera OD increased slightly with *M. leprae* as the antigen, but remained at a relatively low level, 0.23 OD units. The difference between pooled control sera and pooled lepromatous sera with *M. smegmatis-A* was approximately four-fold as compared to *M. leprae,* where the difference between both pools was approximately eight-fold.

The reactivity of *M. leprae* is compared to autoclaved *M. leprae* in Table 4. Autoclaving *M. leprae* increased its reactivity in the ELISA, but did not result in an increase in the difference of reactivity between con-

TABLE 4. *Comparison of reactivity of patient sera to autoclaved* M. smegmatis, M. leprae, *and autoclaved* M. leprae *as measured by ELISA.*

Sera	*M. smeg.*-A[a]	*M. leprae*	*M. leprae*-A
Lepromatous pool[b]	0.60[c]	1.89	1.91
LL patients			
002	0.55	1.38	1.54
003	0.82	1.95	1.96
011	0.45	1.21	1.34
TT patients			
012	ND[d]	0.21	0.31
013	ND	0.29	0.46
Controls[e]	0.14	0.23	0.43

[a] The concentration of cells in the volatile coating buffer was adjusted to an OD of 0.07 at 420 nm for each antigen preparation.

[b] The lepromatous pool represents pooled sera from seven individuals.

[c] ELISA OD units were read at 492 nm.

[d] ND = not done, previous tests of this serum resulted in values which did not differ significantly from control sera.

[e] Control sera represent a pool of sera from seven individuals who were skin test negative for tuberculosis and had not lived in an endemic area for leprosy.

TABLE 5. *Comparison of serological reactivity of* M. smegmatis *and* M. leprae *as measured by ELISA.*

	M. smegmatis	*M. leprae*			
Antigen dilution[a]	None	1:2	1:4	1:10	1:20
Pooled sera[b]					
Lepromatous	0.76[c]	1.36	1.00	0.71	0.37
Controls	0.20	0.27	0.17	0.11	0.05

[a] Starting concentration cells = 0.15 OD units at 420 nm per ml of coating buffer.

[b] Lepromatous sera represent a pool from seven patients. Control sera represent a pool from seven individuals who were skin test negative for tuberculosis and had not lived in endemic areas for leprosy.

[c] ELISA OD units read at 492 nm.

DISCUSSION

In this study we have presented data on the development of an ELISA system which uses, as an antigen, whole cells coated with a volatile buffer and dried onto microtiter plates. We have examined several strains of non-*M. leprae* mycobacteria and two strains of cornyebacteria, which serologically cross-react with mycobacteria. These strains were selected on the basis of their availability, growth characteristics, and previously demonstrated reactivity with sera from leprosy patients ([1, 2, 8, 9]). Of these organisms, it was found that *M. smegmatis* was the most reactive with lepromatous sera (Table 1). The high reactivity of *M. smegmatis* whole organisms in immunoassay has been also noted by Dr. K. Nelson and colleagues, University of Illinois (personal communication). Although heating may have destroyed potentially interesting heat-labile antigens on *M. smegmatis*, serological reactivity was further enhanced by autoclaving (Table 2) prior to coating the organism to microtiter plates. The autoclaving of the cells did not result in morphological changes as seen through the light microscope. Thus the treatment did not seem to interfere with the whole cell presentation of antigen to the microtiter plate or the antibody. Although not proven, we assume that heating removed a mycoside-like material from the surface of the organisms in a way that was similar to the heat induced loss of a surface electron-

trol sera and lepromatous sera. The autoclaving of *M. leprae* resulted in increased background which can be seen by comparing the control sera OD of *M. leprae* and *M. leprae-A*, 0.23 units and 0.42 units respectively. Of the two active tuberculoid (TT) patient sera tested, neither *M. leprae* nor *M. leprae-A* reacted above the background levels of the "natural antibody" in the control sera.

The ELISA antigen reactivities of *M. leprae* and *M. smegmatis-A* were compared by antigen titration (Table 5). Starting with equal coating concentrations as determined by OD at 420 nm, *M. leprae* were diluted and the reactivity of each dilution compared with undiluted *M. smegmatis-A*. It was found that a 1:10 dilution of *M. leprae* in coating buffer was comparable to *M. smegmatis-A* as determined by measuring reactivity to pooled lepromatous sera. The measurement for the *M. smegmatis-A* was 0.76 OD units and for *M. leprae* 0.71 units at a 1:10 dilution. Based on ELISA reactivity the *M. leprae* as coating antigen were 4–10 times more reactive.

transparent zone from *M. lepraemurium* reported by Draper and Rees ([4]). We postulate that the loss of this zone may have exposed a surface component such as an arabinogalactan which was more easily recognized by antibody in sera from lepromatous patients (Figs. 1a and 1b) ([2, 5]). Interestingly, autoclaving *M. leprae* did not result in the enhanced reactivity in the ELISA previously seen with *M. smegmatis*. One explanation could be that the loss of this zone occurred during the extraction procedures used to isolate *M. leprae* from armadillo tissues. Another could be that these antigenic determinants exposed after heating of *M. smegmatis* were already available prior to heating *M. leprae*.

From the data reported here and work in progress, we have found background levels of "natural antibodies" to mycobacteria, corynebacteria, *Pseudomonas,* and common enteric bacteria in all individuals. The ranges for these antibody levels at a serum dilution of 1:500 in our ELISA was 0.10 to 0.40 OD units. In contrast, when testing for "natural antibody" with *Brucella,* a genus not normally seen by the general population, the ELISA levels were below 0.1. Armed with this information we have established control sera to be used as a baseline or blank for our assay system. We have also established positive sera controls from our biopsy-certified LL patients. The range of reactivity of these pooled sera can be seen in Table 3.

As can be seen in Figures 1a and 1b, the spectrum of serological reactivity of classified leprosy patients and control sera parallels the results found with the RIA developed by Harboe, *et al.* ([6]). No advantage was found in using either the IgG or the combined IgM, IgA and IgG conjugates in the ELISA. These findings are similar to those reported by Touw, *et al.* ([11]) and Melsom, *et al.* ([10]). The reactivity of lepromatous sera to several different species of mycobacteria and corynebacteria seen in Table 1 is not unusual since these organisms are known to share common antigenic determinants ([2, 8]). It is known that chronic infections or hyperimmunizations frequently result in production of antibody to common antigenic determinants which masks the reactivity to more specific determinants. Since the ELISA is easily adapted to new antigens, it will be more useful in the future

to use specific antigens for chronic diseases like leprosy, such as the antigen reported by Hunter and Brennan ([3, 7]). The application of this type of antigen has inherent value in eliminating the possibility of the crossreactivity found in sera from patients who have diseases produced by bacteria sharing common antigens or determinants with *M. leprae*. We are currently evaluating the activity of Hunter and Brennan's phenolic glycolipid in our ELISA system.

When we compared the relative reactivity of *M. leprae* to that of *M. smegmatis*-A by way of antigen dilution in the coating buffer, we found that *M. leprae* were more reactive. The reason for this increased serological reactivity was not established. It could have been due to expression of more determinants, different antigenic determinants, or stronger binding of the organisms to microtiter plates. These possibilities are being investigated. Although less *M. leprae* were required for antigen coating, autoclaved *M. smegmatis* were almost as effective in discriminating between control and lepromatous sera. It seems reasonable that autoclaved *M. smegmatis* may serve as an alternative to *M. leprae* in the initial development of ELISA systems. This would be especially true where availability of *M. leprae* and the ease of preparation were primary considerations. Once established the ELISA can then be easily adapted to more specific antigens as they become available.

SUMMARY

An ELISA system was developed for detection of antibodies in leprosy using whole cells of bacteria as an antigen. Whole cells of *M. smegmatis, M. vaccae, M. scrofulaceum, M. leprae, C. diphtheriae,* and *C. xerosis* were compared. *M. smegmatis* was the most reactive against lepromatous sera with OD_{492} readings 1.5 times and five times higher than the others. In addition, when *M. smegmatis* were coated to microtiter plates with a volatile ammonium acetate/carbonate buffer and air dried, the antigen coating was found to be three times more reactive than antigen coated with nonvolatile Na borate buffer. Autoclaving *M. smegmatis* increased the reactivity with lepromatous sera 1.4- to 2.3-fold. *M. leprae* was found to be 4–10 times more reactive than autoclaved *M. smegmatis*. Autoclaving *M. leprae* did not increase reactivity.

Antibody titers of some lepromatous sera had endpoint titers of greater than 1:10,000. Both antihuman IgG and antihuman IgA, IgM, and IgG combined conjugates were found to be equally effective in detecting high levels of antibody in patients with multibacillary diseases.

RESUMEN

Se desarrolló un sistema "ELISA" para la detección de anticuerpos en lepra usando bacterias totales como antígeno. Se compararon los resultados obtenidos con *M. smegmatis, M. vaccae, M. scrofulaceum, M. leprae, C. diphtheriae* y *C. xerosis.* El *M. smegmatis* fue el más reactivo con los sueros lepromatosos, con lecturas (D.O. 492) entre 1.5 y 5 veces más altas que las obtenidas con los otros. Además, cuando *M. smegmatis* se usó para forrar las microplacas con un regulador volátil (acetato de amonio/carbonato) el cual posteriormente se dejó secar al aire, se encontró una reactividad tres veces mayor que cuando el forramiento se hizo con antígeno suspendido en regulador de borato de sodio (no volátil). Sometiendo al autoclave al *M. smegmatis,* la reactividad con sueros lepromatosos se incrementó de 1.4 a 2.3 veces. El *M. leprae* fue de 4 a 10 veces más reactivo que el *M. smegmatis* sometido al autoclave. El someter al autoclave al *M. leprae* no incrementó su reactividad. Los títulos de anticuerpo en algunos sueros lepromatosos fueron mayores de 1:10,000. Tanto los conjugados anti-IgG como los anti-IgA, IgM e IgG, fueron igualmente efectivos en la detección de niveles elevados de anticuerpo en pacientes con la enfermedad multibacilar.

RÉSUMÉ

Un système ELISA a été mis au point pour la détection des anticorps dans la lèpre, au moyen de cellules entières de bactéries utilisées comme antigènes. On a comparé des cellules entières de *M. smegmatis, M. vaccae, M. scrofulaceum, M. leprae, C. diphteriae,* et *C. xerosis. M. smegmatis* s'est révélé être l'organisme qui réagissait le plus activement à l'égard des échantillons de sérums provenant de malades lépromateux, fournissant des lectures O.D.$_{492}$ une 1.5 à 5 fois plus élevées que les autres organismes. De plus, lorsque *M. smegmatis* était appliqué à des plaques de microtitrages, avec un tampon ammonium acétate/carbonate et de l'air sec, le revêtement antigène s'est révélé trois fois plus actif que celui revêtu d'un tampon non volatile de borate de soude. Lorsque *M. smegmatis* était autoclavé, la réactivité à l'égard d'échantillons de sérums lépromateux a été augmenté de 1.4 à 2.3 fois. On a observé que *M. leprae* était 4 à 10 fois plus actif que *M. smegmatis* autoclavé. Le fait d'autoclaver *M. leprae* n'a pas accru sa réactivité. Les titres d'anticorps de certains des échantillons de sérums lépromateux fournissaient des titres extrêmes dépassant 1:10,000. Tant les IgG antihumaines, que les conjugués combinés anti-humains IgA, IgM et IgG, se sont révélés aussi éfficaces pour détecter des niveaux élevés d'anticorps chez les malades atteints d'une affection multibacillaire.

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