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Sensitivity and Specificity of the FLA-ABS Test for Leprosy in Mexican Populations[1]

Maria Eugenia Amezcua, Alejandro Escobar-Gutiérrez, Elvira Mayén, and Jose Victor Cázares[2]

Several serological methods have been proposed for the early diagnosis of leprosy. Among them, the FLA-ABS test, developed by Abe ([1, 2]), has been widely used ([3–5, 9, 11, 12, 14]). In spite of the isolation of the phenolic glycolipid specific for *Mycobacterium leprae* which has proved to be an excellent antigen for research and diagnostic applications ([18]) and some adverse opinions against the FLA-ABS test ([15]), we still consider this method to be a simple and convenient technique for immunoepidemiological leprosy detection and control programs.

The FLA-ABS test is an indirect immunofluorescence technique in which the antigen is whole *M. leprae.* The previous absorption of the sera with BCG, *M. vaccae,* cardiolipin, and lecithin has proved to be successful for the purposes of the test. Nevertheless, with other methods it has been possible to detect remaining crossreacting antibodies against extracts of some mycobacteria ([10]).

With this background and taking into account the mixed ethnic origin of the Mexican Mestizo population ([13]) and the particular distribution of environmental mycobacteria ([6]), we started a study to evaluate the FLA-ABS test with regard to specificity, sensitivity, predictability, and the possibility of finding crossreactions. We found the FLA-ABS test to be a very useful and promising method for epidemiological studies in Mexico.

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[2] M. E. Amezcua, M.Sc., Head, Laboratory for Mycobacterial Research; A. Escobar-Gutiérrez, Ph.D., Head of Department; E. Mayén, B.Sc., Biologist; J. V. Cázares, Tech. Biology Laboratory Technician, Departamento de Investigaciones Inmunológicas, Instituto de Salubridad y Enfermedades Tropicales, Secretaría de Salud México, Carpio 492, México 17, D.F., México.

MATERIALS AND METHODS

Subjects

Venous blood specimens were obtained from six different groups as described below. In all cases, the selection was made taking into account a similar ethnic background (Mestizo) as well as social and economic status, in addition to the main clinical characteristics of each group. The sera were separated under aseptic conditions, transported cold, and stored at −20°C until used. The groups studied were: a) 60 healthy individuals from Mexico City, a nonendemic area; b) 57 patients without any infectious disease from a general hospital also in Mexico City; c) 72 patients with active pulmonary tuberculosis from another Mexico City general hospital; d) 26 healthy individuals from the state of Sinaloa, an endemic area, without any known contact with leprosy patients; e) 100 patients with clinical, bacteriological, histopathological, and immunological diagnoses of lepromatous leprosy (LLp) from the states of Sinaloa and Jalisco; and f) 123 household contacts of LLp patients without any symptom of leprosy from the states of Sinaloa and Jalisco.

Mycobacterial suspensions

Different species of mycobacteria were used: *M. leprae* as antigen, and BCG and *M. vaccae* for serum absorption, and several mycobacteria for evaluation of crossreacting antibodies.

M. leprae. *M. leprae* was obtained from infected armadillo liver, supplied by Dr. J. Convit, Instituto Dermatológico Venezolano, Caracas, Venezuela, and Dr. E. E. Storrs, Medical Research Institute, Florida Institute of Technology, Melbourne, Florida, U.S.A. The bacteria were obtained from the liver by the method of Draper ([7]), and the acid-fast bacilli (AFB) were enumerated as described by Shepard ([17]). The suspension was diluted with phosphate-buffered

saline (PBS), pH 7.2, to a concentration of 1 to 1.5 × 10^8 AFB/ml. The final suspension was aliquoted and stored at −70°C until used.

BCG vaccine. BCG vaccine was prepared by the Instituto Nacional de Higiene, Mexico City, Mexico. For absorption, 1 g of lyophilized bacilli was suspended in 20 ml of PBS, pH 7.2, and dispersed by sonication (sonicator by Heat Systems Ultrasonic, Inc., Plainview, New Jersey, U.S.A.) for 5 min until an homogeneous suspension was obtained, and then stored at 0°–5°C.

M. vaccae. *M. vaccae* was supplied by Dr. Silvia Giono, Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Mexico City, Mexico. For absorption, the mycobacteria were cultivated in Sauton's medium at 37°C for 2 weeks, collected by centrifugation, and washed three times with PBS, pH 7.2. Finally, 1 volume of pelleted bacilli was suspended in 9 volumes of PBS and stored at 0°–5°C.

Mycobacteria suspensions. For investigation of crossreacting antibodies, BCG (Instituto Nacional de Higiene), *M. fortuitum, M. chelonei, M. kansasii,* and *M. vaccae* (Dr. Silvia Giono) were cultivated in Löwenstein-Jensen medium until a luxurious growth was achieved. The suspension was made by scraping the slope of the medium with 5 ml of PBS, pH 7.2. *M. lepraemurium* (Hawaiian strain) was maintained in outbred white mice (CFW strain), and obtained from heavily infected livers by the Prabhakaran method ([16]). All mycobacterial species used were dispersed to homogeneous suspensions by sonication. The suspensions were diluted to a final concentration of 1 to 1.5 × 10^8 AFB/ml with PBS, pH 7.2. Smears were prepared in the same way as indicated for the FLA-ABS test.

Reagents

Carbon tetrachloride. A special reagent grade of carbon tetrachloride (E. Merck AG, Darmstadt, West Germany) was used for pretreatment of specimens.

Trypsin. A 1% solution was prepared from trypsin 1:250 (Difco Laboratories, Detroit, Michigan, U.S.A.) in 0.2 M Tris-HCl buffered saline, pH 8.0. At the time of use, this solution was diluted 1:10 with the same buffer.

Cardiolipin-lecithin solution. A cardiolipin-lecithin solution in absolute ethanol (each one at 0.4%) (Roche Laboratories, Inc., Loganville, Georgia, U.S.A.) 1:20 dilution with PBS (diluent A) was used for the absorption of sera. The solution used to dilute the absorbed sera was prepared with 9 volumes of diluent A mixed with 1 volume of 1% bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Missouri, U.S.A.) solution (diluent B).

Goat anti-human globulin fluorescein labeled antibody (Becton, Dickinson Immunocytometry Systems, Mountain View, California, U.S.A.). Before its use, absorption was done by adding an equal volume of BCG suspension (5% w/v). After incubation at 37°C for 30 min, the mixture was centrifuged at 1500 × *g* × 30 min at 4°C. The supernatant was filtered through a membrane filter of 0.22 μ pore size in order to remove any suspended particles. This solution was used as a secondary antibody in the test.

Buffered glycerol. Buffered glycerol was prepared with 1 volume of freshly prepared carbonate buffer, pH 9.5, and 9 volumes of glycerol (reagent grade nonfluorescent; Merck).

FLA-ABS test

Smears of *M. leprae*. Five μl of the *M. leprae* suspension containing 1.5 × 10^8 bacilli/ml was spread in a circle 1 cm in diameter marked on a glass slide used for immunofluorescence tests (M6177; Scientific Products Div., McGaw Park, Illinois, U.S.A.). The smear was air dried with a hair dryer.

Pretreatment of smears. To eliminate remaining lipids, the smears were immersed in carbon tetrachloride at room temperature for 10 min. Contaminating armadillo tissue was digested with 0.1% trypsin solution at 37°C for 30 min. Before use, the slides were washed three times with PBS, pH 7.2, 5 min each time.

Absorption of test serum. Two-tenths ml of the test serum, 0.2 ml of the BCG suspension, 0.2 ml of the *M. vaccae* suspension, and 1.4 ml of diluent A were mixed together. The mixture was incubated at 37°C for 30 min and centrifuged at 1500 × *g* ×

THE TABLE *Results of the FLA-ABS test in leprosy and nonleprosy cases.*

Group	Description	Cases	
		Positive/total	% Positive
1	Healthy individuals from nonendemic area	0/60	0
2	Cases from general hospitals in nonendemic area	0/57	0
3	Patients with active pulmonary tuberculosis	0/72	0
4	Healthy noncontacts from endemic area	0/26	0
5	Patients with lepromatous leprosy (LLp)	99/100	99
6	Household contacts	108/123	87.8

30 min. The supernatant was filtered through a 0.22 μ pore size membrane filter.

Primary reaction. The smears of *M. leprae* were covered with the absorbed serum and incubated at 37°C for 1 hr in a moist chamber, and were then washed three times with PBS, pH 7.2. The positive sera were titrated, making fourfold dilutions up to 1:10,240 with diluent B.

Secondary reaction. The smears were covered with the anti-human globulin fluorescent antibody solution and incubated at 37°C for 1 hr or at 4°C overnight in a moist chamber. Washing was done as described, and the smears were allowed to dry.

Control sera. After the first screening with sera from LL patients, high-titer positive samples were selected and pooled. This pool was used as a positive control. PBS, pH 7.2, and a pool of sera from healthy individuals from nonendemic areas without any history of leprosy contact were used, both as negative controls.

Reading. The smears were mounted with carbonate-buffered glycerol, pH 9.5, and a coverslip. Reading was done at magnification ×600 with a fluorescent microscope (Immunopan; American Optical Corp., Buffalo, New York, U.S.A.), and no less than 50 fields were observed and counted in each smear.

Abe's original criteria classified the results in a scale from 0 to 4, where 2 to 4 are positive and the rest are negative ([2]). For the purpose of this work, we made no attempt to use this classification, and we only reported positive reactions when a strong characteristically green fluorescence of fluorescein isothiocyanate (FITC) was observed in all or in the majority of bacilli, with the remaining showing a weak fluorescence. Negative reactions were reported when it was not possible to detect green fluorescence bacilli or their quantity outnumbered the fluorescent ones.

Statistical analysis. In order to evaluate the specificity, sensitivity, and negative and predictive values of the FLA-ABS test, the calculations suggested by Galen and Gambino ([8]) were applied.

RESULTS

The results of the FLA-ABS tests with sera from the six groups studied are summarized in The Table. Negative results were found in all of the samples from groups 1 to 4. In group 5, all except one had a positive reaction (99%). In the household contacts from LLp patients (group 6), 87.8% showed positive reactions regardless of the extent or degree of the contact with the case.

The Figure shows a four-squared diagram which includes a comparison of the results of the FLA-ABS test with those from conventional criteria for leprosy diagnosis and calculations for sensitivity, specificity, and negative and positive predictive values of the test. The results from groups 1 to 4 were considered as a whole for the known negative cases.

In order to test residual crossreactivity of the absorbed sera with common environmental mycobacteria, 20 randomly chosen absorbed sera from LLp cases were tested in the same way as the FLA-ABS method with other mycobacteria instead of *M. leprae*. The selected species were the three most frequently found infecting the Mexican population even without any clinical disease ([6]): *M. fortuitum, M. cheloni,* and *M. kansasii* in addition to BCG, *M. vaccae,* and *M. lepraemurium.* Negative results were found in all cases using both 1:10 and 1:40 sera dilutions.

EVALUATION OF FLA-ABS TEST

EPIDEMIOLOGICAL AND CLINICAL DATA	Positive	Negative	
Positive	True positive (A) 99	False negative (B) 1	Sensitivity (A/A + B) 0.99
Negative	False positive (C) 0	True negative (D) 189	Specificity (D/C + D) 1
	Predictive value of positive (A/A + C) 1	Predictive value of negative (D/B + D) 0.99	

THE FIGURE. Evaluation of the FLA-ABS test.

DISCUSSION

The results described here demonstrate the specificity and sensitivity of the test as performed under our conditions. The easiest way to measure the test's specificity is to consider the number of false-positive results, i.e., the number of positives among individuals lacking the disease or a known contact with patients. In this work, these cases were composed of all subjects belonging to groups 1 to 4, and because no sample from them gave a positive reaction, the test had an index of specificity of 100%. Nevertheless, we may not exclude the possibility of finding, in a larger sample, some positive results in persons without a history of the disease or known contact with leprosy cases as has been reported ([2]).

Frequently results comparing sick and healthy populations give significant differences because the authors really analyze different populations in regard to the ethnic background and living conditions. For this reason, we made a careful selection of groups 1 to 4, trying to match them as closely as possible to leprosy cases in regard to their ethnic origin and social and economic status. Thus, we can conclude that positive reactions, except in leprosy cases or household contacts, must be a very infrequent finding, at least in Mexico.

In order to calculate the index of sensitivity, it is necessary to consider the number of false-negatives, that is, those cases having a negative result in the presence of the disease. Here we found a value of 99% because only one LLp patient did not give the expected positive result. This patient had been diagnosed 40 years ago, and had started treatment at that time. At the time of the study all his lesions were healed, and he had a negative bacterial index. Thus, the test appears to give little, if any, false-negative reactions in LLp cases.

The potential usefulness of the test cannot be evaluated just from the values of specificity and sensitivity. It must take into account the positive and negative predictive values of the test which are in relation to the prevalence of the disease. Thus, the high levels of confidence of the positive and negative results we found are a direct consequence of the low prevalence of leprosy in Mexico (0.21/1000 inhabitants). Even considering only those individuals from an endemic area (group 4), the absence of false-positive results strengthens the importance of this test as a tool for detection of contact with leprosy bacilli.

The major problem when looking for antibodies against a specific mycobacterium is the wide distribution of other phylogenetically related bacteria with crossreacting antigens which can be found in different ecological niches, both environmental or symbiotic with humans. For this reason, the elimination of antibodies induced by mycobacteria other than *M. leprae* is an essential procedure for the FLA-ABS test. The method performed in this work to verify the absence of crossreactivities proves the efficacy of the materials used for absorption. The mycobacteria chosen are the main

species isolated from the Mexican population, and they also represent a good group of crossreacting antigens. It is of special interest that the BCG used for the absorption of sera is the same strain employed for vaccination in Mexico. As was pointed out in our Results section, we did not find any false-positive reactions. In this regard, the report of Gillis, *et al.* ([10]) must be carefully interpreted, because they used antigens prepared in a different way than for the FLA-ABS test and selected another method which does not necessarily have to be related with immunofluorescence.

The results of group 6 (household contacts of patients) show that after a continuous and close contact with a bacilli-spreading case, there is a high possibility of developing a good specific antibody response. However, the demonstration of the presence of these antibodies does not mean that there is a simultaneous occurrence of a subclinical *M. leprae* infection or a higher risk to develop a serious form of leprosy. In order to establish a diagnosis, additional data must be obtained from other immunological studies, such as a Mitsuda intradermal test or some *in vitro* evaluation of specific T-cell response. Thus, household contacts with a positive FLA-ABS test and negative results in the Mitsuda or *in vitro* test could be the ones who are likely to develop lepromatous leprosy. In a prospective study now under way we are testing this hypothesis, looking for the frequency of the emergence of clinical manifestations of leprosy in a group of this kind in comparison with household contacts of the same patients with an adequate, specific, cellular immune response.

SUMMARY

The epidemiological surveillance for leprosy must include several clinical and laboratory procedures. The FLA-ABS test of Abe could be a useful tool for this purpose because it allows the demonstration of an effective contact with *Mycobacterium leprae*. In order to establish the specificity, sensitivity, and predictability of the FLA-ABS test under Mexican conditions, we studied sera collected from six groups of individuals: 60 healthy donors from a nonendemic area, 57 cases hospitalized for conditions other than infectious diseases from a general hospital in a nonendemic area, 72 patients with active pulmonary tuberculosis, 26 healthy individuals from an endemic area, 100 patients with polar lepromatous leprosy (LLp), and 123 household contacts of patients with LLp. The FLA-ABS test was negative with sera from the first four groups. Strong positive reactions were found in all LLp patients except one; the false-negative results could be attributed to successful treatment and a long-standing cure in this patient.

Analysis of these results shows 100% specificity, 99% sensitivity, and predictability values of the test of 100% for positive results and 99% for negative ones. In addition, none of the 20 randomly selected sera from LLp patients were positive with crossreacting mycobacteria. Because 87.8% of the household contacts were positive in the absence of clinical manifestations of leprosy, it is possible to conclude that a positive result by itself is not enough to establish an early diagnosis of the disease, especially among inhabitants of endemic areas. Probably this test, together with another that could measure the specific cellular immune response, could be useful in detecting infected individuals at risk of developing serious forms of leprosy. Further work in this area is now in progress.

RESUMEN

El estudio epidemiológico de la lepra debe incluir varios análisis clínicos y de laboratorio. La prueba del anticuerpo fluorescente absorbido (FLA-ABS) de Abe podría resultar útil para este propósito porque permite la demostración de un contacto efectivo con el *Mycobacterium leprae*. Para establecer la especificidad, la sensibilidad y la predictibilidad de la prueba FLA-ABS bajo condiciones mexicanas, se estudiaron los sueros colectados de 6 grupos de individuos: 60 donadores sanos de un área no endémica, 57 casos hospitalizados por causas no infecciosas de un hospital general de un área no endémica, 72 pacientes con tuberculosis pulmonar activa, 26 individuos sanos de un área endémica, 100 pacientes con lepra lepromatosa polar (LLp), y 123 contactos convivientes de pacientes con LLp. La prueba FLA-ABS fue negativa con los sueros de los primeros cuatro grupos. En todos los pacientes con LLp (excepto en uno) se encontraron reacciones fuertemente positivas. El resultado falso negativo se podría atribuir a lo exitoso del tratamiento que condujo a una curación duradera en este paciente.

El análisis de estos resultados muestra un 100% de

especificidad, un 99% de sensibilidad, y una predictibilidad del 100% para los resultados positivos y del 99% para los negativos. Además, ninguno de los 20 sueros de pacientes con LLp seleccionados al azar dió un resultado positivo con micobacterias de reacción cruzada. Puesto que el 87.8% de los contactos convivientes fueron positivos en ausencia de manifestaciones clínicas de la lepra, es posible concluir que un resultado positivo per se no es suficiente para establecer un diagnóstico temprano de la enfermedad especialmente entre los habitantes de zonas endémicas. Probablemente esta prueba junto con alguna otra que pudiera medir la respuesta inmune celular, podría ser útil en la detección de los individuos infectados en riesgo de desarrollar formas serias de la lepra. Un trabajo de este tipo está ahora en desarrollo.

RÉSUMÉ

La surveillance épidémiologique de la lèpre doit faire appel à plusieurs méthodes cliniques et de laboratoire. L'épreuve FLA-ABS de Abe est utile à cet égard, car elle permet de mettre en évidence des antécédents de contact avec *Mycobacterium leprae* ayant résulté en une infection. En vue de mesurer la spécificité, la sensibilité, et le caractère prédictif de l'épreuve FLA-ABS dans le contexte du Mexique, on a étudié des échantillons de sérum récoltés chez six groups d'individus: 60 donneurs sains habitant une région non endémique, 57 malades hospitalisés dans un hôpital général de région non endémique, pour des affections non infectieuses, 72 malades souffrant de tuberculose pulmonaire active, 26 personnes saines habitant en région endémique, 100 malades de la lèpre présentant une lèpre lépromateuse polaire (LLp), et 123 contacts domiciliaires de malades atteints de lèpre lépromateuse polaire. L'épreuve FLA-ABS s'est révélée négative pour tous les échantillons de sérum provenant des quatre premiers groupes. Des réactions fortement positives ont été observées chez tous les malades LLp, sauf un; ce dernier résultat faussement négatif pourrait être attribué à un traitement efficace, qui avait été suivi chez ce malade d'une guérison prolongée.

L'analyse de ces résultats permet d'attribuer à cette épreuve une spécificité de 100%, une sensibilité de 99%, et un caractère prédictif à 100% pour les résultats positifs, et à 99% pour les résultats négatifs. De plus, parmi 20 échantillons de sérum choisis au hasard chez des malades LLp, aucun n'a été trouvé positif pour des mycobactéries à réactivité croisée. Comme 87,8% des contacts domiciliaires se sont révélés positifs en l'absence de toute manifestation clinique de lèpre, on peut conclure qu'un résultat positif ne suffit pas à lui seul pour poser un diagnostic précoce de lèpre, particulièrement chez des habitants des régions endémiques. Cette épreuve pourrait dès lors être utile pour détecter des individus infectés, et donc exposés au risque de développer une forme sévère de la maladie, si elle était utilisée conjointement à une autre épreuve qui pourrait mesurer la réponse immunitaire cellulaire spécifique. Les travaux continuent dans ce sens.

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