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Enzyme-Linked Immunosorbent Assay for Serological Diagnosis of *Nocardia brasiliensis* and Clinical Correlation with Mycetoma Infections

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We previously identified three immunodominant antigens obtained from a *Nocardia brasiliensis* cell extract and recognized by sera from mycetoma patients (M. C. Salinas-Carmona, L. Vera, O. Welsh, and M. Rodríguez, Zentralbl. Bakteriol. 276:390–397, 1992). In the present work, we obtained a crude extract from a mass culture of *N. brasiliensis* HUJEG-1 and purified two immunodominant antigens, the 26- and 24-kDa proteins, by using simple physicochemical techniques. With these antigens, we developed a conventional solid-phase enzyme-linked immunosorbent assay and tested 30 serum samples from mycetoma patients, 29 from tuberculosis patients, 24 from a leprosy group, and 31 from healthy individuals. Our results show for the first time statistically significant differences in serology among these groups. All mycetoma patients with a positive culture for *N. brasiliensis* had absorbance values higher than 0.3. On the other hand, the mycobacterium-infected patients as well as the healthy individuals all had absorbance values below that level. Moreover, we found a close correlation between the clinical condition of the mycetoma patients and the anti-26- and anti-24-kDa protein antibody concentrations. We therefore propose the use of this assay in routine clinical laboratories to confirm the diagnosis of *N. brasiliensis* infection in human mycetoma cases. In addition, the possible application of this assay in the serodiagnosis of *Nocardia asteroides* infection is also discussed.

Nocardia brasiliensis and *Nocardia asteroides* are gram-positive bacteria that produce different infections in humans. The former is frequently localized, and the latter is often systemic. *N. brasiliensis* is usually responsible for a lesion involving the skin and adjacent tissues and known as mycetoma (15). *N. asteroides* is the etiologic agent of human nocardiosis and may infect the lungs and the central nervous system, producing abscess (6, 17). In both mycetoma and nocardiosis cases, the diagnosis is made on the basis of clinical findings and confirmed with microbiological cultures that can take more than 2 weeks because of the slow rate of growth of these microorganisms. Serological detection of nocardial infection was attempted, with inconclusive results, in part because of the low sensitivity of the immunodiffusion techniques used and the lack of specificity of the antigen mixtures, producing extensive cross-reactivity with sera from mycobacterium-infected patients (3, 11). However, more recently, Sugar et al. described two immunodominant antigens from an *N. asteroides* culture filtrate (19), these proteins later being used in a dot blot assay and an enzyme immunoassay with high sensitivity and specificity (1, 2). El-Zaatari et al. also reported apparently good results with a Western blot (immunoblot) assay and semipurified antigens instead of whole bacterial antigen mixtures (7). In 1990, Boiron and Provost (4) used an affinity chromatography column to purify *N. asteroides* antigens for a Western blot assay and found little cross-reactivity with *N. brasiliensis*, *Rhodococcus rhodochrous*, and *Mycobacterium tuberculosis*. In contrast to these cited studies on *N. asteroides*, there has been little information on the use of serology for the routine clinical laboratory analysis of *N. brasiliensis* infections (23).

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We recently demonstrated the presence of three immunodominant antigens in a crude extract from *N. brasiliensis* by using a Western blot assay. These antigens were identified by use of sera from mycetoma patients; however, a weak reaction or no reaction with sera from mycobacterium-infected individuals was found (16). We were able to isolate and partially purify two of the immunodominant antigens by using simple techniques (21). In this work, we describe an enzyme-linked immunosorbent assay (ELISA) that uses the 26- and 24-kDa proteins for the serological diagnosis of *N. brasiliensis* infections and show its usefulness in assessing the response to treatment.

MATERIALS AND METHODS

Antigen preparation. We used *N. brasiliensis* HUJEG-1 to obtain a crude extract as previously described (16), with a few modifications. We inoculated a unicellular suspension of *N. brasiliensis* into 1-liter Erlenmeyer flasks containing brain heart infusion medium (Difco Laboratories, Detroit, Mich.) and incubated them for 7 days at 37°C without agitation. Bacteria were harvested, washed with distilled water three times, treated two times with an ethanol-ether (1:1 the first time and 1:3 the second time), mixture, and dessicated under vacuum and the cells were ground in a mortar with glass powder. Disrupted bacteria were suspended in 0.01 M Tris-HCl (pH 7.4) with 0.01 M magnesium acetate, and the suspension was magnetically stirred overnight at 4°C. Intact cells were removed by centrifugation at 1,200 × *g* for 15 min, and the supernatant was ultracentrifuged at 144,000 × *g* for 3 h and dialyzed for 24 h at 4°C against distilled water. The yellowish clear supernatant was lyophilized and stored at −20°C.

Antigen purification. We isolated two of the immunodominant antigens from the *N. brasiliensis* crude extract in two

steps. First, we eliminated the bands that cross-reacted with mycobacterial antigens by ammonium sulfate precipitation as follows. The *N. brasiliensis* crude extract (100 mg) was dissolved in 12 ml of phosphate-buffered saline (PBS) (pH 7.2) and slowly mixed with 12 ml of a saturated ammonium sulfate solution, previously adjusted to pH 7.2 with ammonium hydroxide. The precipitate was discarded after centrifugation at 1,500 × *g* for 30 min, and the supernatant was dialyzed against normal saline solution for 4 days at 4°C and lyophilized. In the second step for 26- and 24-kDa protein isolation, we reconstituted the lyophilized sample with 1 ml of pH 7.2 PBS and incubated it with 150 μl of DNase I (Sigma Chemical Co., St. Louis, Mo.) at 1 μg/mg of protein and 37°C for 2 h. The sample was centrifuged at 1,500 × *g* for 15 min, dialyzed against distilled water for 24 h at 4°C, and applied to a Sephadex G-100 column (23 by 2.5 cm) previously equilibrated with pH 7.2 PBS, and the flow rate was adjusted to 0.5 ml/min with the same PBS as an elution buffer. We collected 2-ml fractions and recorded their A_{280}s on a PMQ3 spectrophotometer (Zeiss). The molecular weight markers used for column calibration included alcohol dehydrogenase (molecular weight, 150,000), carbonic anhydrase (29,000), and cytochrome *c* (12,400), all obtained from Sigma.

PAGE analysis of the immunodominant antigens. To analyze the fractions eluted from the column, we used polyacrylamide gel electrophoresis (PAGE) under denaturing conditions with sodium dodecyl sulfate (SDS). The Laemmli discontinuous buffering system (12) was used, with an 8 to 18% gradient resolving gel and a 5% stacking gel. Electrophoresis was run at 150 V until the dye reached the bottom, and the gels were stained with Coomassie blue R-250. The molecular weight standards included bovine serum albumin (molecular weight, 66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and lactalbumin (14,000), all obtained from Bio-Rad Laboratories, Richmond, Calif.

Western blot identification of the immunodominant antigens. Further characterization of the *N. brasiliensis* antigens isolated as described here was carried out by an immunoelectrotransference assay. In brief, after electrophoresis was completed as described above, the proteins were transferred to nitrocellulose membranes (0.45-μm-pore size) with a Trans-blot cell (Bio-Rad) for 90 min at 240 V; 25 mM Tris–192 mM glycine (pH 8.3) buffer and 20% methanol were used as described by Towbin et al. (20). The nitrocellulose strips were then blocked with 3% fish gelatin in pH 7.2 PBS for 60 min at 37°C. Serum samples from patients and controls were diluted 1:25 with 1.5% gelatin in PBS-Tween 20 (1:1,000) and individually incubated with the nitrocellulose strips with gentle agitation for 2 h. After five washes, an anti-human immunoglobulin G–peroxidase conjugate (Sigma) was added. The chromogen solution contained diaminobenzidine (Sigma) in PBS and 0.2% hydrogen peroxide.

Human serum samples. Peripheral blood from 26 patients with a clinical diagnosis of mycetoma and a positive culture for *N. brasiliensis* was collected. We also included three patients with actinomycetoma caused by *N. asteroides* and one patient with a positive culture for *Actinomadura madurae*. We also tested sera from patients with a confirmed diagnosis of leprosy and attending the Dr. José E. González University Hospital. Serum samples from tuberculosis patients with a positive sputum culture for *M. tuberculosis* were kindly donated by Manuel Díaz-Rodríguez and Roberto Mercado-Longoria. Control sera were obtained from healthy individuals.

Enzyme immunoassay (ELISA). Flat bottom, 96-well, polystyrene plates from Costar were used in the ELISA. We used the method described by Engvall and Perlmann (8) with some modifications. Purified antigen (0.5 μg per well) containing the 26- and 24-kDa proteins was incubated at 4°C overnight. After three washes with pH 7.2 PBS-Tween 20 (1:1,000), blocking was done with skim milk at a 5% concentration in the PBS solution. The plate was washed five times with the washing solution mentioned above and incubated at 37°C for 1 h with patient and control sera diluted 1:500. After a wash, 200 μl of goat anti-human immunoglobulin G conjugated to peroxidase per well was added, and incubation was continued at 37°C for 1 h. The chromogen substrate solution was composed of *o*-phenylenediamine and hydrogen peroxide; 1 N sulfuric acid was used as a stop reagent. The A_{492} was read with a semiautomatic ELISA plate reader.

The specificity of the assay described here was evaluated by comparing the absorbance values for sera from confirmed tuberculosis, leprosy, and *N. brasiliensis*-infected patients. The results were statistically analyzed by use of the Student *t* test. To assess the sensitivity of the test described here, we diluted sera from five mycetoma patients to 1:500, 1:1,000, 1:2,000 and 1:4,000, analyzed their absorbance values by linear regression, and determined the significance of the correlation coefficient by using a *t* test. Intra-assay reproducibility was evaluated with triplicates of different samples during the same test, and results were statistically analyzed by use of the mean and standard deviation. Inter-assay reproducibility was determined on 3 consecutive days with the same serum samples.

RESULTS

***N. brasiliensis* crude antigen.** A humid bacterial mass (100 g) was harvested from Erlenmeyer flasks; the yield of crude protein antigen, as determined by the method of Lowry et al. (14), was 190 mg. After precipitation with ammonium sulfate at 50% saturation, we eliminated some bands that cross-reacted with tuberculosis and leprosy sera. Figure 1 shows a gel with the crude antigen, the protein bands precipitated with 50% saturation ammonium sulfate, and the bands that remained in the supernatant, from which the 26- and 24-kDa proteins were later isolated. These two proteins could not be separated by gel electrophoresis because of their similar molecular weights.

Immunodominant antigen purification. Figure 2 shows a gel with the crude antigen and some fractions eluted from the Sephadex G-100 column and stained with Coomassie blue R-250, demonstrating the presence of a single band of approximately 26 to 24 kDa. However, when using a more sensitive method to analyze these fractions, i.e., the Western blot assay (Fig. 3), we detected the presence of two close bands whose relative mobilities corresponded to those of the 26- and 24-kDa proteins; in addition, a minor, low-molecular-weight contaminant was evident when the hyperimmune serum of a rabbit immunized with the crude antigen from *N. brasiliensis* was used. This contaminant did not react with the sera from the patients included in this study.

Anti-*N. brasiliensis* antibodies determined by the ELISA. Using the semipurified 26- and 24-kDa proteins as antigens in the ELISA described here, we found that 26 patient serum samples from the *N. brasiliensis*-infected group had absorbance values equal to or above 0.3. In contrast, serum samples from the 53 mycobacterium-infected individuals, including both the tuberculosis and the leprosy patients, always showed absorbance values below this level, as dem-

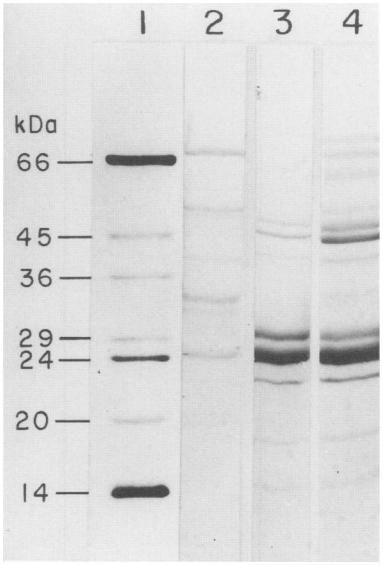

FIG. 1. SDS-PAGE analysis of the *N. brasiliensis* cell extract in an 8 to 18% gradient gel stained with Coomassie blue R-250. Lanes: 1, molecular mass markers; 2 and 3, precipitate and supernatant, respectively, of the *N. brasiliensis* cell extract treated with ammonium sulfate; 4, crude cell extract.

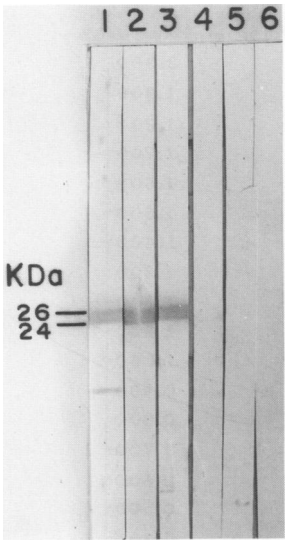

FIG. 3. Western blot analysis of the purified immunodominant antigens from the *N. brasiliensis* cell extract. Lanes: 1, serum from a rabbit immunized with the *N. brasiliensis* cell extract; 2 and 3, sera from mycetoma patients; 4, serum from a leprosy patient; 5, serum from a tuberculosis patient; 6, serum from a healthy control.

onstrated in Fig. 4. Of the 30 mycetoma cases, 29 had serum samples that showed values above the cutoff of 0.3, and only 1 serum sample, that from a patient with mycetoma caused by *A. madurae*, showed a value below this level, and this value was close to the borderline. In the healthy control group, one serum sample showed an absorbance value above 0.3; this sample was from a graduate student who had been working for 3 years in the isolation and purification of *N. brasiliensis* antigens and who is clinically normal.

Correlation between anti-*N. brasiliensis* antibody concentration and clinical condition. We had the opportunity to test five mycetoma patients during, before, and after anti-*N. brasiliensis* therapy and found that in all cases, during active disease, the absorbance values were always above 0.3. After the medical treatment was completed and the patients were classified as cured, the absorbance values fell below this level. Moreover, two patients had a reactivation of their lesions, and their sera showed an increase in absorbance values (Table 1).

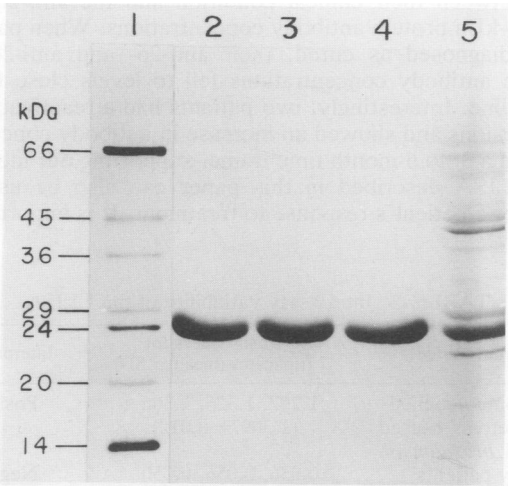

FIG. 2. SDS-PAGE analysis of the *N. brasiliensis* cell extract after ammonium sulfate precipitation and purification by gel filtration chromatography. Lanes: 1, molecular mass markers; 2, 3, and 4, fractions eluted from the Sephadex G-100 column; 5, *N. brasiliensis* cell extract.

Sensitivity and reproducibility of the ELISA. To estimate the intra-assay variability of the ELISA, we included triplicates of different samples as well as normal controls. We summarize our findings in Table 2, which shows representative results demonstrating the highly consistent data according to the mean ± standard deviation for each group. The day-to-day variation was evaluated by testing the same samples on different days; the results of a typical experiment, expressed as the mean ± standard deviation, are shown in Table 3 and demonstrate that there was hardly any variation, reflecting the same antibody concentrations. The sensitivity of the ELISA was tested with dilutions of 1:500, 1:1,000, 1:2,000 and 1:4,000 of five different serum samples from mycetoma patients with a positive culture for *N. brasiliensis*. Table 4 shows that the correlation coefficient (linear regression) was above 0.94, on the basis of a 1:500 serum dilution, and that the *t* test *P* value was <0.05 in a comparison of the 1:500 and 1:4,000 serum dilutions.

DISCUSSION

N. asteroides infections are increasingly being reported in AIDS patients (10) and other immunocompromised patients (5, 13, 18). The epidemiology of these infections in undeveloped countries is not known. On the other hand, mycetoma caused by *N. brasiliensis* is known to be endemic in India, Sudan, and some countries of Latin America (9). For both *N. asteroides* and *N. brasiliensis* infections, the diagnosis is

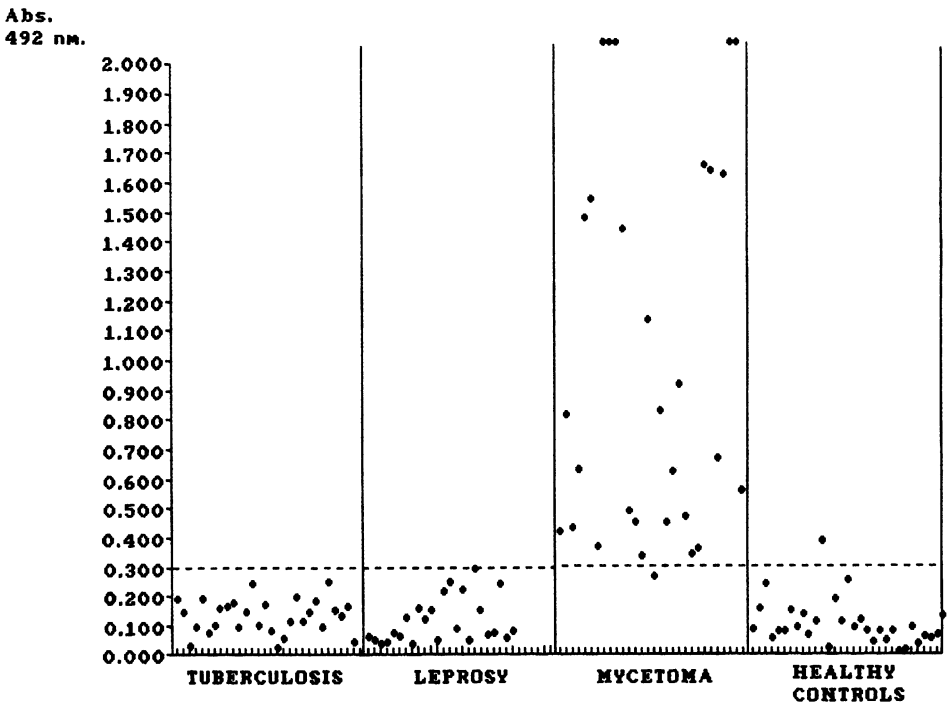

FIG. 4. Anti-*N. brasiliensis* antibody concentrations, determined by the ELISA, in sera from tuberculosis, leprosy, and mycetoma patients and normal individuals. Abs., absorbance. $P < 0.001$.

usually difficult and is based on clinical findings and the identification of the etiologic agent in culture. The slow rate of growth of these microorganisms and the need for invasive procedures to collect a culture sample, especially in nocardiosis cases, stress the need for a serological test. The serodiagnosis of nocardial infections has been traditionally unsuccessful, in part because of the low sensitivity of the tests used, such as the immunodiffusion tests used by Humphreys et al. (11) and by Blumer and Kaufman (3); another problem is the extensive cross-reactivity among mycobacteria and nocardiae (22, 23).

TABLE 1. Relationship between clinical condition of mycetoma patients and anti-*N. brasiliensis* antibody concentration, determined by the ELISA

Patient	Clinical condition	Absorbance
1	Active	0.457
	Cured	0.106
2	Active	2.00
	Cured	0.067
3	Active	0.858
	Cured	0.109
	Reactivated	0.380
4	Active	0.814
	Cured	0.203
	Reactivated	1.104
5	Active	1.479
	Cured	0.138

The ELISA described here helped in the routine clinical laboratory confirmation of the *N. brasiliensis* etiology of mycetoma cases because all active cases had positive absorbance values. Our finding that a case of mycetoma caused by *A. madurae* was correctly classified as negative also indicates the utility of this ELISA and provides additional support for its specificity.

We had the opportunity to include in our study five patients with mycetoma caused by *N. brasiliensis* before, during, and after their treatment and found a close correlation between their clinical condition and the anti-26- and anti-24-kDa protein antibody concentrations. When patients were diagnosed as cured, their anti-26- and anti-24-kDa protein antibody concentrations fell to levels close to the borderline. Interestingly, two patients had a reactivation of their lesions and showed an increase in antibody concentrations in a 3- to 6-month time frame, supporting our idea that the ELISA described in this paper can also be used to monitor a patient's response to treatment. It is important to

TABLE 2. Intra-assay variability of the ELISA

Serum source	Absorbances for triplicates (mean ± SD)	Interpretation
Mycetoma patients (mycetoma caused by *N. brasiliensis*)	1.297, 1.326, 1.306 (1.309 ± 0.012)	Positive
Leprosy patients	0.056, 0.056, 0.050 (0.054 ± 0.003)	Negative
Tuberculosis patients	0.056, 0.058, 0.060 (0.058 ± 0.002)	Negative
Healthy controls	0.029, 0.031, 0.023 (0.027 ± 0.004)	Negative

TABLE 3. Inter-assay variability of the ELISA[a]

Serum source	Absorbances for triplicates (mean ± SD)[b]	Interpretation
Mycetoma patients (mycetoma caused by *N. brasiliensis*)	1.740, 1.858, 1.866 (1.866 ± 0.130)	Positive
Leprosy patients	0.127, 0.157, 0.133 (0.138 ± 0.014)	Negative
Tuberculosis patients	0.063, 0.059, 0.064 (0.062 ± 0.002)	Negative
Healthy controls	0.031,0.028, 0.039 (0.032 ± 0.005)	Negative

[a] Anti-*N. brasiliensis* antibody concentrations were determined on different days.
[b] The three values were determined on days 1, 2, and 3 and are listed in that order.

mention that this ELISA differentiates sera from tuberculosis- and leprosy-affected individuals from those of *Nocardia*-infected subjects. The specificity of the test is not 100%, because of cross-reactivity between *N. asteroides* and *N. brasiliensis*, and we found that three patients with mycetoma caused by *N. asteroides* had positive results; however, this finding was not a disadvantage, because *N. asteroides* rarely produces skin lesions. Moreover, it is highly likely that this test will be of help in the serodiagnosis of patients infected with *N. asteroides*. More studies of nocardiosis patients are needed to evaluate the possible usefulness of this test in the diagnosis of systemic infections caused by *N. asteroides*, taking advantage of the cross-reactivity of the anti-26- and anti-24-kDa protein antibodies.

TABLE 4. Evaluation of ELISA sensitivity and statistical analysis of results

Patient	Serum dilution	Absorbance	Statistical correlation coefficient (*r*)
1	1:500	1.868	0.979491
	1:1,000	1.452	
	1:2,000	1.128	
	1:4,000	0.838	
2	1:500	0.664	0.948651
	1:1,000	0.561	
	1:2,000	0.397	
	1:4,000	0.288	
3	1:500	0.500	0.965748
	1:1,000	0.388	
	1:2,000	0.242	
	1:4,000	0.157	
4	1:500	0.503	0.988406
	1:1,000	0.344	
	1:2,000	0.189	
	1:4,000	0.137	
5	1:500	0.594	0.967448
	1:1,000	0.459	
	1:2,000	0.276	
	1:4,000	0.185	

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