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Isolation and Purification of Two Immunodominant Antigens from *Nocardia brasiliensis*

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Two immunogenic proteins from a crude extract of *Nocardia brasiliensis* were purified to homogeneity. A 61-kDa protein (P61) was isolated from a 50% ammonium sulfate precipitate in two steps. Initially, P61 was obtained by electroelution in a 10% nondenatured preparative polyacrylamide gel electrophoresis (PAGE). In a second step, the eluate from the nondenatured gel was run in a 12% sodium dodecyl sulfate (SDS) preparative polyacrylamide gel. After elution, a single band was demonstrated by SDS-PAGE and Western blot (immunoblot). Also, a 24-kDa immunogenic protein (P24) was isolated by gel filtration in a Sephadex G-100 column and then by electroelution in a 12% nondenatured polyacrylamide gel. In a previous paper, we showed by Western blot assays that these proteins are recognized by the sera of mycetoma patients and not by sera from mycobacterial-infected or healthy individuals. We consider these proteins to be good candidates for the study of the host-parasite relationship in nocardial infections. The possible clinical application of these purified antigens in a serological diagnosis is discussed.

Mycetoma is a chronic granulomatous disease that affects the skin and underlying tissue including bone and sometimes adjacent organs (20, 24). In Mexico, most of the cases are produced by actinomycetes, and *Nocardia brasiliensis* is the most commonly isolated agent (9). *Nocardia asteroides* also produces mycetoma and is the main etiologic agent of pulmonary nocardiosis, which causes about 500 to 1,000 cases per year in the United States (4, 22). Although the pathogenic mechanisms of nocardial infections have not been completely elucidated, some authors have reported experiments indicating that the cellular immune response plays an important role in resistance (10, 12). The role of the humoral immune response in nocardial infections is not well known, although some authors have observed that antibody production is associated with worsening lesions (18). The majority of the studies on the host-parasite relationship in nocardial infections have used whole bacterial cells without determining the individual antigens that elicit such responses. However, in the case of *N. asteroides*, El-Zaatari et al. have recently reported that three proteins obtained from a culture filtrate extract from this microorganism reacted in a Western blot (immunoblot) assay with sera from patients with nocardiosis (11). In addition, Sugar et al. identified by Western blot two immunodominant antigens from a culture filtrate of *N. asteroides* with approximate molecular masses of 55 and 31 kDa (23). These two proteins reacted specifically with sera from patients with nocardiosis and demonstrated a low cross-reactivity with sera from patients with tuberculosis (3, 8).

In the case of *N. brasiliensis*, an antigenic composition analysis is not complete. However, Jimenez et al. have reported the production of monoclonal antibodies against a crude extract from *N. brasiliensis*, although the nature of the involved antigens was not determined (13).

In a previous study (21), we found that sera from patients with actinomycetoma caused by *N. brasiliensis* recognized several proteins from a crude extract in a Western blot assay. The sera reacted more intensively and specifically with three bands of approximate molecular masses of 61, 26, and 24 kDa. On the basis of these results, we think that these proteins are of interest for the study of the immune response in *Nocardia* infections. The purpose of this article is to describe the methodology used to isolate and purify the 61- and 24-kDa immunodominant proteins from *N. brasiliensis* by using classic physicochemical methods.

MATERIALS AND METHODS

***N. brasiliensis* strain.** The strain used in this study (HU JEG-1) was obtained from the Departmento Microbiología, Facultad de Medicina, Universidad Autonoma de Nuevo Leon. This bacterium was isolated at the José Eleuterio Gonzalez University Hospital from a mycetoma case and was maintained by subculturing on Sabouraud agar. The strain was identified by conventional methods in our laboratory and was kindly confirmed by L. Ajello at the Centers for Disease Control, Atlanta, Ga.

Bacterial crude extract. Since the inoculation of a liquid medium with fragments of colonies of *N. brasiliensis* produces a low bacterial mass, we used unicellular suspensions to inoculate the Erlenmeyer flasks. With this technique, we obtained higher quantities of CFU and an increase in bacterial mass. The unicellular suspensions were prepared as follows: *N. brasiliensis* HUJEG-1 was grown for 48 h in 125-ml Erlenmeyer flasks with 30 ml of brain heart infusion (Difco Laboratories, Detroit, Mich.) at 37°C in a water bath with constant agitation. The organisms were harvested by centrifugation and washed three times with a sterile saline solution. The pellet was disaggregated at low speed in a motor-driven, sealed container for 1.5 min (Eberbach, Ann Arbor, Mich.). By this method, the bacterial aggregates were disrupted into individual cells and short filaments that were separated from the large fragments by differential centrifu-

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gation as described by Beaman and Maslan (5). The suspension was adjusted to an approximate optical density at 580 nm of 1.0 in a PMQ3 spectrophotometer (Zeiss, Germany). To obtain bacterial mass, we inoculated 0.5 ml of this unicellular suspension into 40 1-liter Erlenmeyer flasks each containing 170 ml of brain heart infusion medium. The flasks were incubated at 37°C without agitation for 7 days. The crude extract was obtained by a modified method based on the one used by Ortiz-Ortiz et al. (17). Briefly, the bacterial mass was harvested by centrifugation, washed with warm distilled water, and defatted with ethanol-ethylic ether (1:1). The bacteria were then desiccated under vacuum, and the powder was suspended in 0.01 M Tris-HCl, pH 7.4, with 0.01 M magnesium acetate. The suspension was sonicated in a Biosonik apparatus (Bronwill Scientific, Rochester, N.Y.) at a 60-probe intensity for 30 min in an ice bath and magnetically stirred overnight at 4°C. The sonicated bacteria were centrifuged at 3,000 × *g* for 15 min to separate fragments and nondisrupted cells, and the soluble fraction was obtained by centrifugation at 144,000 × *g* for 3 h at 4°C in a L8-70M ultracentrifuge (Beckman, Palo Alto, Calif.). The clear supernatant was dialyzed at 4°C for 24 h against distilled water, by using a dialysis sack (Sigma Chemical, Co., St. Louis, Mo.) with a molecular weight cutoff of 12,000 to 14,000. After dialysis, the solution was lyophilized and stored in aliquots at −20°C until used. The protein content was determined by the Lowry method (16) by using bovine serum albumin (Sigma) as a standard.

PAGE analysis of the crude extract from *N. brasiliensis*. To analyze the crude extract, we used a gradient 8 to 18% polyacrylamide resolving gel and a 4% stacking gel (10 cm by 12 cm by 0.75 mm) with the Laemmli buffering system (15). A sample of 25 μg was boiled for 2 min in a buffer solution containing a final concentration of 2.5% sodium dodecyl sulfate (SDS), 2.5% (vol/vol) 2-mercaptoethanol, 12.5% sucrose, and bromophenol blue as the tracking dye. Ten microliters was applied to the gels, and polyacrylamide gel electrophoresis (PAGE) was carried out at 150 V for the resolving gel until the dye reached the front. Molecular weight standards were obtained from Sigma; they included bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate-dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200). The gels were stained with silver nitrate (Bio-Rad Laboratories, Richmond, Calif.) or with Coomassie blue R-250.

Salting-out fractionation of the crude extract. The crude extract from *N. brasiliensis* was precipitated at different ammonium sulfate concentrations (20, 50, 70, and 80%). Precipitates were suspended in a minimal volume of isotonic saline solution and extensively dialyzed against saline-borate solution, pH 8.4. After dialysis, precipitates were analyzed by SDS-PAGE by using the electrophoresis technique described above.

We precipitated the *N. brasiliensis* crude antigen with ammonium sulfate at 50% saturation in preparative assays because under these conditions P61 and P24 can be obtained with fewer contaminant bands. Briefly, 100 mg of crude extract was dissolved in 12 ml of phosphate-buffered saline (PBS), pH 7.2, and an equal volume of saturated ammonium sulfate solution was added slowly with gentle stirring agitation. The precipitate was resuspended and dialyzed against 8 liters of saline solution. The dialyzed precipitate was analyzed by SDS-PAGE in a 12% polyacrylamide gel, and the protein content was determined by the Lowry method.

Isolation of P61 in native form. A sample of the crude extract from *N. brasiliensis* was precipitated with ammonium sulfate at 50% saturation and mixed with sample buffer (without SDS and 2-mercaptoethanol) at a 3:1 dilution and applied to a preparative PAGE system (3 mm, thickness) with a 10% running gel and a 5% stacking gel. The electrophoresis buffer was 384 mM glycine–250 mM Tris. The system was run at 150 V for approximately 4 h at 4°C. After electrophoresis, the position of P61 in the unstained gel was determined easily because this protein had a greenish color. This fraction was excised from the gel and electroeluted in an ISCO (Lincoln, Nebr.) concentrator by using the electrophoresis buffer mentioned above as the electrode buffer and a 1:10 dilution of the same buffer in water in the electroelution cup. A dialysis membrane with a molecular weight cutoff of 12,000 to 14,000 (Sigma) was used to trap the eluted proteins. The electroelution was carried out at 120 V for 2 h at 4°C, and the proteins were recovered from the cathodic chamber. The purity was assessed by SDS-PAGE, and due to the expected low amount of pure protein in the eluate, we used the Coomassie blue binding method to determine the protein content (6).

Purification of *N. brasiliensis* P61. An aliquot of the eluted material obtained as described in the previous section was resolved by SDS-PAGE and showed the 61-kDa band plus two or three minor contaminants of lower molecular masses. To further purify P61, we applied this eluate to a preparative SDS-PAGE system and recovered the protein by electroelution. The position of P61 in unstained gels was previously determined by electrophoresing a sample of crude antigen precipitated with ammonium sulfate at 50% saturation in an analytical SDS-PAGE gel at 12% polyacrylamide. The relative mobility of the protein was calculated and compared with the migration of prestained proteins (Bio-Rad) run next to the antigen lane. The prestained proteins used as calibrators were phosphorylase *b* (molecular weight, 130,000), bovine serum albumin (75,000), ovalbumin (50,000), carbonic anhydrase (39,000), soybean trypsin inhibitor (27,000), and lysozyme (17,000) (Bio-Rad). The eluates obtained in the preparative assays were analyzed by SDS-PAGE and stained with Coomassie blue R-250 or silver nitrate or electrotransferred to assess the purity.

Isolation of P24 by gel filtration chromatography. By using 40 mg of the *N. brasiliensis* crude extract to isolate P24, this starting material was dissolved in 800 μl of PBS and incubated at 37°C with DNase I (1 μg/mg of protein; Sigma) for 2 h. The sample was centrifuged, and the supernatant was applied to a Sephadex G-100 (Sigma) column (40 by 1.6 cm). The flow rate was adjusted to 0.5 ml/min, and the sample was eluted with PBS, pH 7.4. Aliquots were collected in 2-ml fractions, and the A_{280} was determined with a PMQ3 spectrophotometer (Zeiss). The absorbances of the aliquots were plotted against their elution volumes. Several samples from the peaks obtained were analyzed by SDS-PAGE to determine the position of P24.

Purification of P24. To increase P24 purity, we used a second purification step. The fractions from the gel filtration column containing isolated P24 were pooled, dialyzed against distilled water, and concentrated by lyophilization. The powder was reconstituted with 1 ml of distilled water and mixed with sample buffer without SDS and 2-mercaptoethanol. This was then applied to a nondenaturing preparative Laemmli system with a 12% running gel and a 5% stacking gel. Electrophoresis was carried out at 150 V until the tracking dye reached the edge of the gel. The migration of P24 was determined by cutting a fraction from the edge of the gel and staining with Coomassie blue. The fraction of the

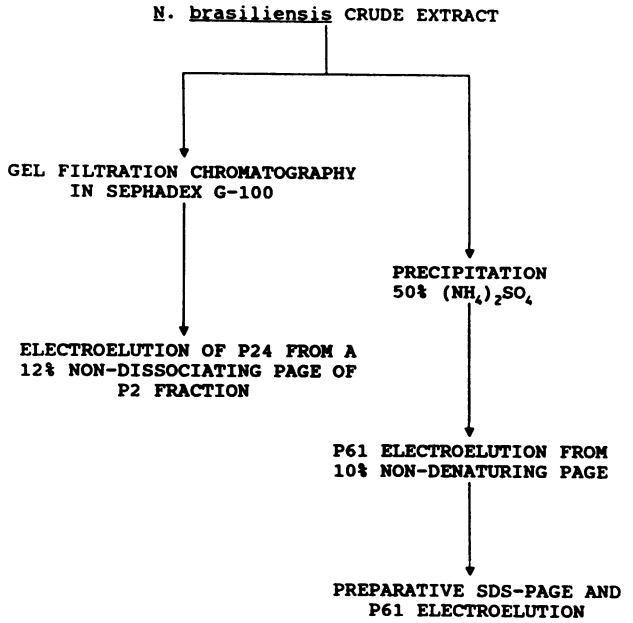

FIG. 1. Flow chart showing the purification procedure for P61 and P24 from *N. brasiliensis* crude extract.

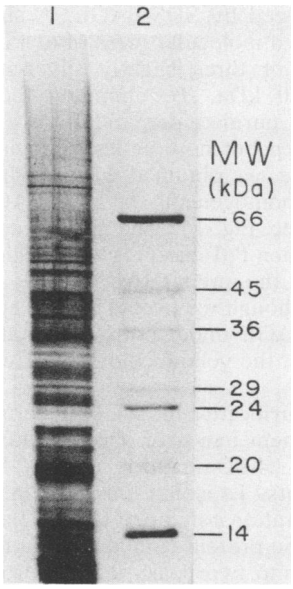

FIG. 2. *N. brasiliensis* antigens analyzed by gradient SDS-PAGE (8 to 18% polyacrylamide). Lane 1, *N. brasiliensis* sonicated extract (25 μg); lane 2, molecular mass markers. Silver nitrate stain was used.

unstained gel containing P24 was removed, and the protein was electroeluted as described above. The eluate obtained was analyzed by SDS-PAGE in a 12% polyacrylamide gel and silver stain. The molecular weights of both purified proteins (P61 and P24) were calculated by determining their relative mobilities in a 12% SDS–PAGE gel and comparing them with those of the molecular weight standards.

Western blot analysis of purified P61 and P24. To assess the purity of P61 and P24, 300 ng of each protein was electrophoresed by gradient SDS-PAGE (8 to 18% polyacrylamide) as described above. After electrophoresis, the proteins from the gel were transferred to nitrocellulose paper (0.45-μm pore size filter; Bio-Rad) in cold transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% [vol/vol] methanol), for 90 min at 240 V in a Trans-blot cell (Bio-Rad). The paper was blocked for 60 min at 37°C with 3% fish gelatin in PBS (Hipure liquid gelatin; Norland Products, Inc., New Brunswick, N.J.) and incubated for 2 h at 37°C with rabbit serum (raised against the sonicated extract from *N. brasiliensis*) diluted 1:500 with 1.5% gelatin in PBS–0.1% Tween 20. The paper strip was washed six times with 0.3% gelatin in PBS–0.1% Tween 20 and incubated with a peroxidase-conjugated anti-rabbit immunoglobulin G (Sigma) for 2 h at room temperature. The paper was washed again and developed with 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 20 ml of PBS plus 60 μl of 33% H_2O_2.

RESULTS

SDS-PAGE analysis of the *N. brasiliensis* extract. Figure 1 shows a summary of the techniques used for the isolation and purification of two immunodominant antigens. The crude extract from *N. brasiliensis* showed a yellowish color and contained approximately 35 bands when analyzed in a silver-stained gradient SDS-PAGE gel, as shown in Fig. 2.

Isolation and purification of the 61-kDa protein from *N. brasiliensis*. Figure 2 shows that the bands in the region of 60 kDa are poorly represented in the crude extract. As a first purification step, we fractionated the extract by precipitating the proteins at different ammonium sulfate concentrations. We observed that, at 50% saturation, P61 precipitated massively; by SDS-PAGE analysis, P61 was observed as the major component of the precipitated proteins (Fig. 3). From this material, we purified P61 in its native state by PAGE in a nondenatured system. In the resulting gels, we found the presence of a yellow band which corresponded to the mobility of P61. After electroeluting this yellow protein, the

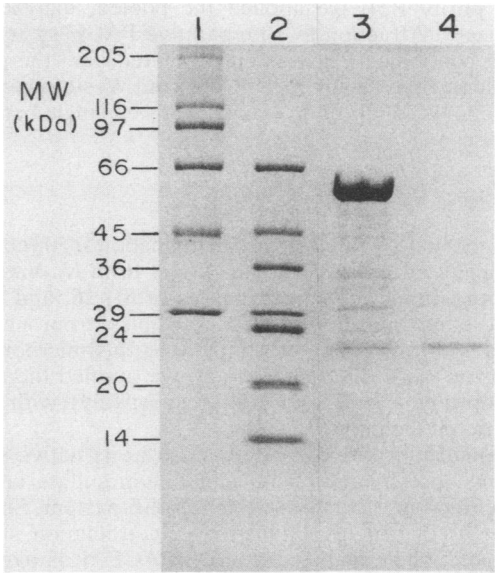

FIG. 3. Gradient SDS-PAGE (8 to 18% polyacrylamide) of isolated proteins. Lanes 1 and 2, molecular mass markers; lane 3, ammonium sulfate precipitate at 50% saturation; lane 4, sample from the second protein peak (P2) obtained by gel filtration of the crude extract in Sephadex G-100. A Coomassie blue-stained gel was used.

eluate was analyzed by SDS-PAGE. A single major band corresponding to a molecular mass of 61 kDa was observed as well as two or three slightly silver-stained bands of approximately 40 kDa. To eliminate these contaminant bands from our purified preparation, we used a second electroelution step but now under dissociating conditions. By this technique, we obtained purified P61 that was demonstrated to be homogeneous by SDS-PAGE and Western blot analyses (see Fig. 5). The yellow color of P61 was observed only when P61 was present at high concentrations, for instance, in the preparative gels. It is important to mention that, although we used a similar amount of sample in preparative PAGE under both nondenaturing and denaturing conditions, the yellow color of P61 was not observed in the latter.

Isolation and purification of P24. In the crude extract, there were two prominent bands of about 20 kDa. One of them proved to be the 24-kDa band recognized by the sera from mycetoma patients. To isolate this protein, in first instance we used a gel filtration column of Sephadex G-100. By using this technique, the protein components of the crude extract were separated into two peaks that absorbed at 280 nm: a high-molecular-weight peak close to the void volume (P1) and a low-molecular-weight peak (P2), with its maximum absorbance near the elution volume of carbonic anhydrase (29 kDa). A third peak (P3) was observed at the bed elution volume, but it contained only the yellowish pigment from the crude extract; the other peaks remained colorless. When we analyzed several aliquots from the peaks by SDS-PAGE, we observed that P1 consisted of almost all the crude extract bands; P3 did not show any band with silver nitrate. The most important finding was that P2 contained only one band of an approximate molecular mass of 24 kDa in the SDS-PAGE gel stained with Coomassie blue or silver nitrate. To check the purity of this preparation, we analyzed by Western blot a sample from pooled P2 that was revealed with rabbit serum against the crude extract. With this more-sensitive method, we observed P24 plus a contaminant band of an approximate molecular mass of 20 kDa (Fig. 4). To completely purify P24, we applied the pooled, dialyzed, and lyophilized P2 fraction to a preparative PAGE system. The fraction containing P24 was electroeluted, and the purified protein was analyzed by SDS-PAGE and Western blot. With both methods, the protein was shown as a single band (Fig. 5).

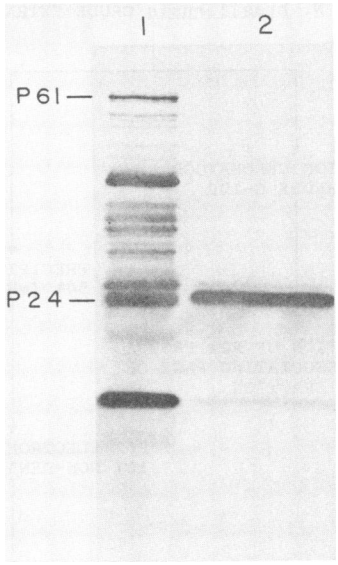

FIG. 4. Electrotransference of isolated P24. Lane 1, crude antigen (25 μg); lane 2, P2 fraction of *N. brasiliensis* crude antigen obtained by gel filtration in Sephadex G-100 (2.3 μg). The paper was developed with a polyclonal rabbit serum raised against sonicated *N. brasiliensis* extract.

DISCUSSION

We reported previously the identification of three immunodominant antigens in a crude extract from *N. brasiliensis* with approximate molecular masses of 61, 26, and 24 kDa that were recognized by serum samples from untreated patients with mycetoma attributable to this microorganism (21). In the work described herein, we purified the 61- and 24-kDa proteins (P61 and P24, respectively) with simple conventional techniques.

We found that P61 can be obtained in its native state in two steps: precipitation with ammonium sulfate and then electroelution in a nondissociating PAGE system. Since the nondenatured protein obtained by electroelution shows a single band when analyzed by SDS-PAGE, it is likely that this protein in its native state is a monomer of 61 kDa or a composite macromolecule with 61-kDa subunits.

This protein shows a yellowish color that facilitates observation of it in unstained preparative gels. Because the crude extract also shows a greenish-yellow color, we concluded that there are two chromogenic substances in the extract: one corresponding to P61 and another component that migrates at the ion front in the SDS-PAGE analysis of the crude extract and is eluted close to the bed volume in the gel filtration column in Sephadex G-100 (P3). It would be of interest to study the biochemical properties of this colored P61 in order to obtain information on the structure and function of this protein in nocardial cells.

The P61 obtained in this first step was not 100% pure, since it showed a slight contamination with other bands of about 40 to 45 kDa. Because of the difference in molecular weight between the contaminants and P61, it was easy to obtain P61 by electroelution in SDS conditions. However, under these circumstances, P61 lost its characteristic yellow color, possibly because of the biochemical changes caused by the treatment with 2-mercaptoethanol and SDS.

P24, the other immunodominant protein purified from the *N. brasiliensis* crude extract, was isolated by gel filtration in Sephadex G-100 in a low-molecular-weight peak (P2). By analysis of the P2 fraction in an SDS-PAGE gel stained with Coomassie blue, a single band corresponding to P24 was observed, but when it was examined by Western blot, a contaminant band was also found. This contaminant band could be a P24 subunit, a degradation fragment produced by the SDS treatment, or perhaps a different protein. This third possibility is strongly supported, since we obtained a single band by analysis with SDS-PAGE after the purification of P24 from P2 by electroelution in preparative PAGE. It is important to mention that P24 was only slightly stained with silver nitrate, perhaps because of the low content of superficial charges that have been associated with the photochemical mechanisms of silver nitrate staining (1). In contrast, it was strongly stained by Coomassie blue R-250.

Because of the well-known antigenic cross-reaction between *N. brasiliensis* and other *Nocardia* species as well as with *Mycobacterium* strains (2, 7, 14, 19), it is possible that the proteins purified in our study share some antigenic

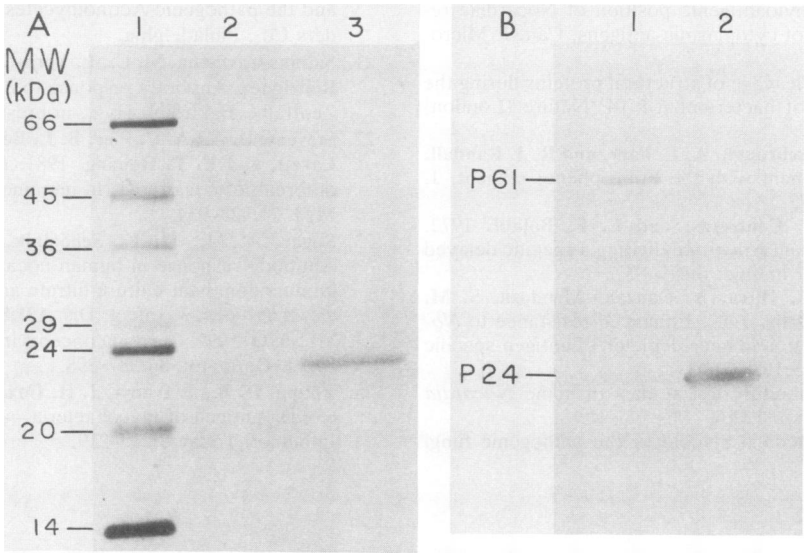

FIG. 5. Analysis of purified P61 and P24. (A) Lineal 12% SDS-PAGE gel stained with silver nitrate and counterstained with Coomassie blue R-250 to detect P24. Lane 1, molecular weight markers; lane 2, P61 from preparative SDS-polyacrylamide gels; lane 3, P24 purified by electroelution from the P2 fraction of the crude extract obtained in Sephadex G-100. (B) Electrotransference of purified proteins to nitrocellulose paper. A rabbit serum raised against the sonicated crude extract was used to detect the transferred antigens. Lanes 1 and 2, same as lanes 2 and 3 of panel A, respectively.

homology with other proteins previously studied. P61 could be related to the 65-kDa protein from *Mycobacterium tuberculosis* that has been demonstrated to share epitopes with some other bacteria, e.g., *N. asteroides*, *Mycobacterium leprae*, and *Escherichia coli* (25). The relationship of the *M. leprae* protein with P61 of *N. brasiliensis* is supported by their close molecular weights and the slight recognition of P61 by sera from leprosy patients in Western blot assays (21).

Since both proteins, P61 and P24, seem to interact strongly with the immune system, their purification will aid in understanding the immune response to nocardia. On the other hand, because of their antigenicity and the low cross-reaction with sera from patients infected with some related microorganisms (mycobacteria), these antigens could be useful in developing a serological assay to detect antinocardia antibodies. This will facilitate the identification of infected patients, as well as the follow-up of their pharmacological treatment.

We decided to purify P61 and P24 because of their ability to induce antibodies in infected human beings. We have not determined whether they stimulate T-cell responses, but we are trying to determine whether P61 or P24 is capable of inducing a cellular immune response in order to provide more information on the immunopathogenic mechanisms of *N. brasiliensis* infection.

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