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Immunochemical and Structural Integrity of Surface Protein Antigens of Mycobacteria During Separation from Armadillo Liver Tissue[1]

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The radical change in *M. leprae* availability in recent years has been the result of the demonstration by Kirchheimer and others that heavy *M. leprae* infections could be established in nine banded armadillos ([10, 15]). This important advancement in *M. leprae* propagation provides a unique opportunity for initiating studies concerned with the antigenic character of *M. leprae*. Our laboratory is interested in the antigenic analysis of *M. leprae* with the specific goal of identifying and characterizing surface antigens since these antigens are likely to interact with the host's immune response. Surface antigens of *M. leprae* may also be specific ([1, 8, 14]) and would therefore be candidate antigens for the serologic diagnosis of subclinical *M. leprae* infections. Antigenic analysis of *M. leprae* requires that the bacilli be purified away from armadillo tissue. This is a formidable task that has been investigated by Dr. Philip Draper of the National Institute for Medical Research, London, England ([4]). Using that procedure, purified bacilli were found to be exceptionally clean as judged by electron-microscopy, and it has been extensively used in the World Health Organization Immunology of Leprosy Program (IMMLEP). This earliest procedure of IMMLEP utilized both nonionic detergent and enzymatic treatment of infected tissue. Since we are interested in surface antigens of *M. leprae*, we chose to investigate whether detergents and proteolytic enzymes might release or destroy these antigens. This report indicates that surface protein antigens of *M. smegmatis* are removed from organisms and at least partly destroyed during various steps that have been frequently employed in the purification of *M. leprae* from infected armadillo tissue.

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MATERIALS AND METHODS

Organisms and culture conditions. *M. smegmatis* ATCC 19420 was obtained from the American Type Culture Collection, Rockville, Maryland. Organisms were grown on Middlebrook and Cohn 7H11 agar base supplemented with OADC enrichment (BBL Div., Becton, Dickinson and Co., Cockeysville, Maryland). Cultures were grown at 37°C with 5% CO_2. Occasionally cultures were grown on a defined 7H9 medium (BBL Div., Becton, Dickinson and Co., Cockeysville, Maryland) to evaluate possible adsorption of media components onto the surface of *Mycobacteria*.

Iodination procedures. The iodination technique used was the lactoperoxidase mediated method described by Hubbard and Cohn [9] for the iodination of red cell membranes. *M. smegmatis* cultured for 72 hours were removed from agar plates with sterile cotton swabs and suspended in cold 0.05 M Na phosphate, pH 7.1–7.2 containing 0.1% Tween-80. Organisms were centrifuged at 10,000 × g for 15 minutes. The washing procedure was repeated three times and the final pellet was resuspended in buffer and the suspension adjusted to an optical density of 0.6 at 548 nm. Ten milliliters of this suspension was centrifuged and the organisms ($2–4 \times 10^9$) were resuspended in one ml of buffer and iodinated. One ml of the iodination suspension contained $2–4 \times 10^9$ organisms, 10 μmoles B-D-glucose, 10 munits of glucose oxidase (Sigma Chemical Corp., St. Louis, Missouri), 5 munits lactoperoxidase and 50–100 μCi carrier free $Na^{125}I$ (New England Nuclear Corp., Boston, Massachusetts). Organisms were iodinated at 37°C for 60 minutes in a shaking water bath with occasional mixing with a vortex mixer. The reaction was terminated by the addition of 10 volumes of cold 0.01 mM $Na_2S_2O_3$. Organisms were centrifuged and washed a minimum of five times in 20 volumes of cold buffer. Iodination of normal armadillo liver proteins was done according to the Chloramine T method of Greenwood, *et al.* [6]. Radioactivity measurements were done in a dual channel Packard auto-gamma scintillation spectrometer (Downers Grove, Illinois).

Polyacrylamide gel electrophoresis. Discontinuous polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was done according to the method of Laemmli [12]. Samples were mixed with an equal volume of sample buffer so that the final mixture contained 0.625 M tris-HCl (pH 6.8), 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. Samples were boiled for five minutes and electrophoresed on 10% polyacrylamide, 0.8% bisacrylamide gel columns (0.6 × 10 cm) pH 8.8. Electrophoresis was carried out at 1.5 mA/gel until the dye marker entered the separating gel at which time the voltage was increased to 5 mA/gel. After electrophoresis, gels were sliced into 2 mm fractions using a Gilson gel slicer and counted. Duplicate gels were stained for protein with Coomassie blue or for carbohydrate using the periodic acid Schiff method described by Fairbanks, *et al.* [5].

Preparation of antiserum. Antiserum was raised to *M. smegmatis* in New Zealand white rabbits. The primary immunization consisted of 4 ml (100 mg/ml wet weight) of organisms washed 4 times with phosphate buffered saline (PBS) injected both intramuscularly (IM) and subcutaneously (SQ). Two ml (1 ml/flank) was injected IM and the remaining 2 ml injected SQ in 0.2 ml quantities in the nuchal and inquinal areas. Rabbits were boosted 30 days later and bled 7–10 days after the booster immunization. Antiserum was tested by rocket immunoelectrophoresis against Triton X-100-EDTA solubilized extracts of *M. smegmatis*. Antiserum prepared by this method precipitated greater than 10 *M. smegmatis* antigens.

Solubilization of proteins from iodinated *M. smegmatis*. Solubilization of ^{125}I labelled proteins for gel electrophoresis analysis was done using 2% SDS, containing 20 mM EDTA in 0.05 M tris-HCl, pH 6.8. Treated organisms were centrifuged at 10,000 × g for 20 minutes and the supernatant retained for analysis. Organisms were also treated with 1% Triton X-100 containing 20 mM EDTA in 0.05 tris HCl, pH 6.8. The 10,000 × g supernatant was used for analysis.

Radioimmune assay. The method for antigen binding was essentially that described by Buchanan, *et al.* [2]. Twenty microliters of Triton X-100 solubilized material from ^{125}I labelled *M. smegmatis* was reacted

TABLE 1. *Lactoperoxidase-mediated iodination of* M. smegmatis *surface proteins.*

Reaction mixture	Radioactivity cpm/10^9 organisms
Complete	11,500,000
Minus lactoperoxidase	46,800
Minus glucose oxidase	92,000

with 10 μl of different dilutions of rabbit anti-*M. smegmatis* serum. A 1:20 dilution of normal rabbit serum was used as a diluent. The reactants were mixed thoroughly and incubated at 4°C for 16 hours. After equilibration, all antibodies were precipitated with goat anti-rabbit immunoglobulin serum. Antibody-bound ^{125}I antigen was precipitated, and the percent binding of ^{125}I labelled antigen was determined after centrifugation at 10,000 × g for five minutes and removal of the supernatant.

Recovery of iodinated *M. smegmatis* during separation of organisms from normal liver tissue. Mixtures of ^{131}I labelled normal armadillo liver homogenate and ^{125}I labelled *M. smegmatis* were used to monitor the earliest IMMLEP purification procedure for the separation of *M. leprae* from infected armadillo liver tissue ([4]). Each enzyme and detergent treatment was performed identically to this published procedure. The isotopically labelled mixture was added to the initial homogenization step. Radioactivity of each isotope was determined at each step in the purification procedure and the percent recovery of the original radioactivity determined.

RESULTS

Surface labelling of *M. smegmatis* by lactoperoxidase and ^{125}I. Table 1 shows that intensive iodination of intact *M. smegmatis* is accomplished using the lactoperoxidase mediated iodination procedure. The iodination reaction was highly specific since less than 1% of the total radioactivity observed in the complete labelling mixture was observed when either lactoperioxidase or glucose oxidase was omitted from the reaction. To determine the number and relative subunit molecular weights of labelled proteins of *M. smegmatis*, labelled organisms were solubilized by SDS and analyzed by SDS gel electrophoresis. Radiogels

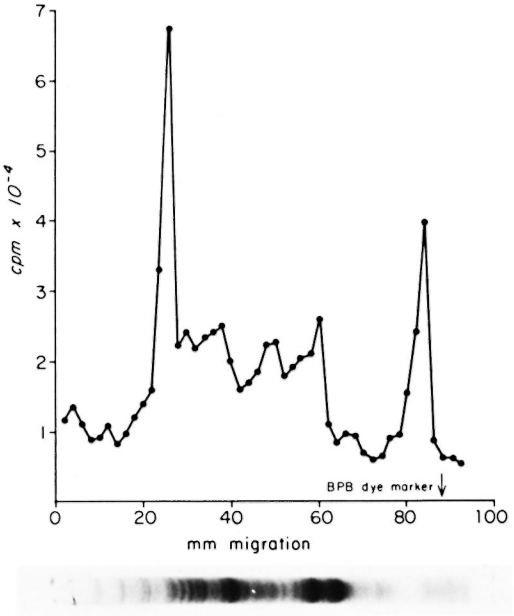

FIG. 1. SDS polyacrylamide gel electrophoresis of SDS solubilized lactoperoxidase mediated ^{125}I labelled *M. smegmatis*. Graph shows radiogel profile. A duplicate gel stained with Coomassie blue is shown below the radiogel graph.

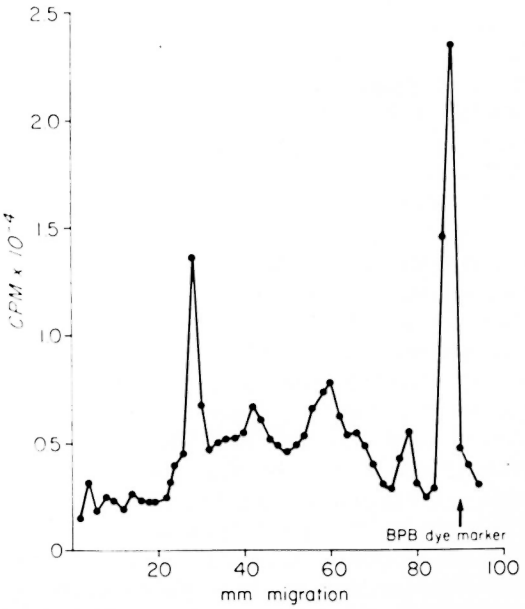

FIG. 2. SDS polyacrylamide gel electrophoresis of Triton X-100 solubilized ^{125}I labelled *M. smegmatis* grown on 7H9 medium without supplement. Note that the radiolabelled protein profile is very similar to the radiogel profile of Triton solubilized *M. smegmatis* grown on supplemented 7H11 medium (see Fig. 1).

TABLE 2. *Separation of* M. smegmatis *from armadillo liver.*

	% original radioactive protein retained	
	^{131}I armadillo	^{125}I *M. smegmatis*
1) Homogenization[1] 500 × g centrifugation in SE buffer[2] supernate retained	91	70
2) 10,000 × g centrifugation sediment retained	40	65
3) Triton X-100-EDTA wash 3×	18	40*
4) Collagenase Rx of pellet, 100 μg/ml pH 7.2, 37°C, 24 hr.	18	40
5) Pronase Rx of collagenase product 100 μg/ml 24 hr., 37°C	18	40
6) Aqueous two-phase partition[3]		
Upper phase (PEG rich)	<2	28 (C) 16 (P)*
Lower & intermediate phase	~20	12 (C) 24 (P)

* Significant loss of *M. smegmatis* surface protein antigens (C) = Collagenase. (P) = Pronase.
[1] Sorvall Omnimixer, 30 ml/10 gm tissue. Top speed 3 min., 4°C.
[2] SE = 0.3 M sucrose, 2.5 mM EDTA, pH 7.2.
[3] 5% polyethylene glycol (PEG 6000), 7% dextran (Pharmacia T500).

of SDS solubilized proteins demonstrated only two major radioactive protein bands (Fig. 1). Since the labelling procedure only labels externally exposed proteins, the peaks shown on the radiogel identify proteins exposed on the surface of *M. smegmatis*. In comparison to the two radiolabelled proteins shown on the radiogel, a duplicate gel stained with Coomassie blue revealed numerous protein bands (Fig. 1). Since a complex growth medium (7H11) was used for the cultivation of organisms, prior to labelling, the possibility existed that protein components of medium origin were being iodinated. To investigate this possibility *M. smegmatis* was grown on the defined medium 7H9 and iodinated using the lactoperoxidase method. As seen in Fig. 2 essentially the same protein labelling was observed when SDS solubilized material was analyzed by SDS polyacrylamide gel electrophoresis. There was, however, a decrease in the slower migrating surface component observed from SDS solubilized organisms grown on the complex medium. It is possible therefore that this component may have been, at least partially, a protein medium constituent. It is possible, however, that since *M. smegmatis* cultivated on 7H9 medium grows poorly compared to organisms grown on the more complex 7H11 medium, that composition may be different with respect to surface structure and function.

Fate of *M. smegmatis* surface proteins during separation from liver tissue. Table 2 demonstrates that less than 2% of isotopically labelled armadillo liver proteins remained associated with *M. smegmatis* organisms after purification. However, we also observed appreciable losses of *M. smegmatis* surface proteins after Triton and pronase treatment (Table 2). Twenty-five percent of the surface labelled protein was lost after Triton treatment. Pronase digestion removed an additional 24% of the exposed protein. These results indicated that separation of *M. leprae* from armadillo tissue using nonionic detergent and proteolytic enzymes may be detrimental for antigenic analysis studies of *M. leprae* bacilli since surface labelled proteins of *M. smegmatis* appear to be selectively removed or destroyed by this procedure. To further investigate the possibility we examined the effect of such treatments on the surface labelled proteins of *M. smegmatis*.

Effect of Triton and pronase on the release of surface protein. Figure 3 shows that both labelled surface proteins that were solubilized using SDS were also released (35–40%) when organisms were treated with 1% Triton X-100. Interestingly, however, the Coomassie blue stained duplicate gel indicated that an additional 20 distinct proteins were solubilized from the organisms by the 60 minutes of incubation with 1% Triton at 37°C. Gels stained for carbohydrate by the PAS procedure demonstrated a single staining band that migrated immediately behind

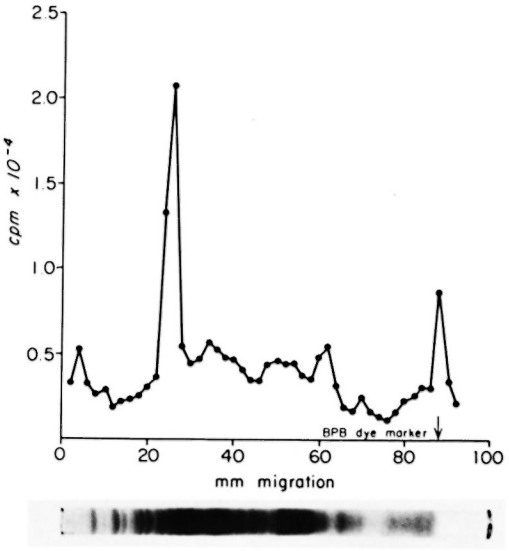

FIG. 3. SDS polyacrylamide gel electrophoresis of Triton X-100 solubilized ^{125}I labelled *M. smegmatis*. Graph shows radiogel profile. A duplicate gel stained with Coomassie blue is shown below the radiogel graph.

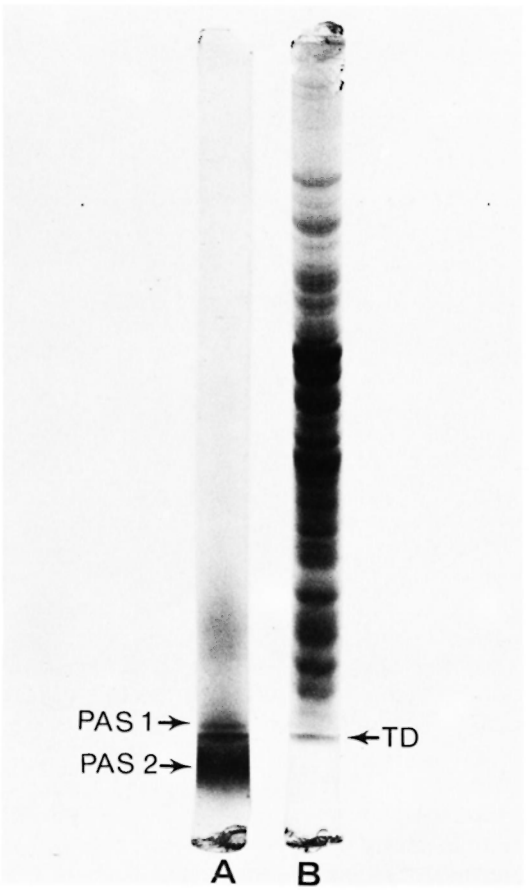

FIG. 4. SDS polyacrylamide gel electrophoresis of Triton X-100, EDTA solubilized *M. smegmatis*. Gel A stained for carbohydrate using the periodic acid-Schiff method. Gel B stained for protein with Coomassie blue. TD = tracking dye; PAS 1—periodic acid-Schiff positive band corresponding to low molecular weight band observed on radiogels; PAS 2—periodic acid Schiff positive band giving diffuse staining reaction.

the gel tracking dye (Fig. 4). The electrophoretic mobility of the PAS positive component was similar to the fast migrating radiolabelled band observed after electrophoresis of Triton solubilized surface labelled material. The component stained weakly for protein but was identifiable in Coomassie blue stained gels. This suggests that the small molecular weight surface protein of *M. smegmatis* is a glycoprotein. The low intensity staining may be in part due to the carbohydrate content of this low molecular weight surface component. An intense PAS positive staining reaction was observed migrating ahead of the tracking dye (Fig. 4). The band was very diffuse, failed to stain for protein and was not iodinated by the lactoperoxidase method. Its PAS positive staining may be due in part to glycolipid ([13]). The treatment of *M. smegmatis* with pronase eliminated the major slow migrating band (Fig. 5) observed by SDS polyacrylamide gel electrophoresis of Triton X-100 solubilized material. In contrast, pronase treatment had no effect on the PAS positive fast migrating band observed from surface labelled organisms. The resistance of the fast migrating component to pronase digestion may be in part due to the presence of carbohydrate protecting the low molecular weight protein from enzyme activity.*

Since our goals are to characterize and purify surface proteins of *M. leprae* as potentially useful serodiagnostic antigens, we examined whether the surface proteins released by Triton were antigens. Figure 6 shows that 56% of the labelled surface proteins of *M. smegmatis* were specifically

* Editor's Note: The author also wishes to acknowledge the work done on the peptidoglycolipids of mycobacteria by Brennan, P. J. and Goren, M. B., Structural studies on the type specific antigens and lipids of the *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium scrofulaceum* serocomplex: *Mycobacterium intracellulare* serotype 9. J. Biol. Chem. **254** (1979) 4205–4211.

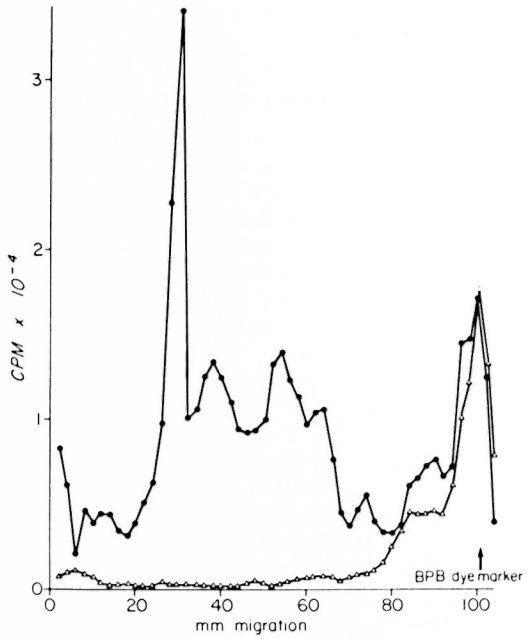

FIG. 5. SDS polyacrylamide radiogel of pronase treated ^{125}I labelled *M. smegmatis*. Triton X-100 extract. ● = control no pronase treatment; △ = 100 μg pronase for 2 hours at 37°C.

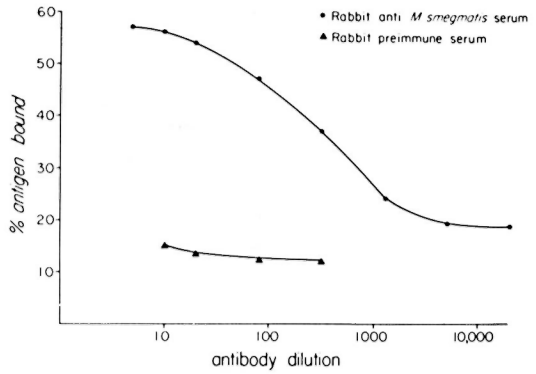

FIG. 6. Antigen binding curve using Triton X-100 solubilized material from ^{125}I surface-labeled *M. smegmatis* organisms.

bound by immune serum as measured by radioimmunoassay, compared to 15% binding produced by preimmune normal rabbit serum. This clearly indicates that the released surface components were antigenic and is consistent with the possibility that one or more of the mycobacterial surface antigens might prove useful in the serodiagnosis of leprosy.

DISCUSSION

The armadillo model ([10, 15]) for the propagation of *M. leprae* provides adequate quantities of the bacillus for antigenic analysis, providing these organisms can be separated with intact antigens from armadillo tissues. Previous procedures ([4]) have purified *M. leprae* remarkably free of host tissue components but may have affected surface proteins of these organisms due to the use of proteases and detergents. Our data are consistent with possible losses of *M. leprae* surface proteins during purification from tissues since lactoperoxidase ^{125}I labelling of surface proteins demonstrated only two proteins exposed on *M. smegmatis* (Figs. 1 and 2), and one of these was sensitive to protease, and both were partially removed by Triton X-100 (Table 2, Figs. 3 and 5). The *M. smegmatis* surface protein of smaller subunit molecular weight was resistant to proteases, perhaps due to the considerable carbohydrate content of this glycoprotein (Fig. 4). Both of these surface proteins were antigenic as indicated by radioimmunoassy (Fig. 6), and this suggests that similar proteins of *M. leprae* might be recognized by the human immune response during leprosy. If similar protein antigens are present in *M. leprae*, improved methods of separating these organisms from armadillo tissues without affecting surface proteins are needed.

Research utilizing *M. leprae* separated from armadillo tissues by procedures that employ proteases and detergents may give spurious results. For example, in a recent study by Harboe, *et al.* ([7]) sonic extracts of *M. leprae* organisms purified by the IMMLEP procedure were found to possess only a limited number of proteins when analyzed by both SDS polyacrylamide electrophoresis and crossed immunoelectrophoresis. In sharp contrast numerous protein antigens were identified from other mycobacterial species using these techniques. Their results are puzzling since care was taken to standardize the protein concentration of the material being analyzed. It is possible that the limited number of recognizable protein antigens of *M. leprae* resulted from prior damage to these protein antigens during purification. Furthermore, no specific *M. leprae* antigens were found. This is in contrast to previous reports of *M. leprae* specific antigens on organisms ana-

lyzed without the use of proteases or detergents ([1,8,14]). These hypotheses are confirmed in the following article ([3]) that identifies a protein antigen extracted from *M. leprae* organisms that appears specific for *M. leprae*. The antigen is precipitated by leprosy patients' sera, implicating its possible importance serodiagnostically. The antigen was extracted from *M. leprae* organisms separated from armadillo liver tissue without the use of nonionic detergents or proteolytic enzyme treatment. It is a protein since its antigenicity is destroyed by proteolytic enzymes or heat treatment. Thus, new improved methods to purify *M. leprae* that preserve this antigen are needed.

SUMMARY

Surface proteins of *Mycobacterium smegmatis* were iodinated using the lactoperoxidase method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated two major surface proteins in the radiolabelled *M. smegmatis*. Both surface proteins were released from *M. smegmatis* using the nonionic detergent Triton X-100. The major surface component was sensitive to pronase digestion and contained no detectable carbohydrate. The second radiolabelled component was found to be of low molecular weight, resistant to pronase digestion and stained positive for carbohydrate by the periodic acid/Schiff method. Triton X-100 solubilized radiolabelled surface proteins were antigenic as assessed by a radioimmune precipitation test. When surface labelled *M. smegmatis* was mixed with armadillo liver tissue and separated from tissue using a method formerly employed by the World Health Organization Immunology of Leprosy Program for the purification of *M. leprae*, as much as 50% of the surface proteins of *M. smegmatis* was either released or destroyed. In addition, another twenty distinct proteins were released from *M. smegmatis* after treatment with Triton X-100. Similar losses of proteins from *M. leprae* may also occur using this procedure for *M. leprae* purification. Separation techniques employing surfactants and enzymatic treatment should be carefully evaluated since proteins lost during these procedures may prove relevant to human immune responses to *M. leprae*.

RESUMEN

Se yodaron las proteínas superficiales del *Mycobacterium smegmatis* usando el método de la lactoperoxidasa. La electroforésis en gel de poliacrilamida con dodecil sulfato de sodio reveló la presencia de dos proteínas superficiales mayores en el *M. smegmatis* marcado radioactivamente. Ambas proteínas se liberaron del *M. smegmatis* por tratamiento con el detergente no iónico Triton X-100. El componente superficial mayor fue sensible a la digestión con pronasa y no contuvo carbohidrato detectable. El segundo componente marcado resultó ser de bajo peso molecular, resistente a la pronasa y se tiñó positivamente para carbohidrato por el método del ácido peryódico de Schiff. Las proteínas superficiales con la marca radioactiva solubilizadas con Triton X-100 fueron antigénicas, según se determinó por una prueba de radioinmunoprecipitación. Cuando se mezcló el *M. smegmatis* yodado superficialmente con tejido hepático de armadillo y posteriormente se separó del mismo usando un método empleado inicialmente en el Programa de la Inmunología de la Lepra de la Organización Mundial de la Salúd para la purificación del *M. leprae*, se encontró una pérdida cercana al 50% de la proteína superficial. El tratamiento del *M. smegmatis* con Triton X-100, condujo a la liberación adicional de otras 20 proteínas distintas. Es posible que el uso de este procedimiento para la purificación del *M. leprae* pueda conducir a pérdidas similares de las proteínas constituyentes. Las técnicas de separación que emplean tratamientos surfactantes y enzimáticos deben ser evaluados cuidadosamente puesto que las proteínas perdidas durante estos procedimientos pueden ser muy relevantes para la respuesta inmune de los humanos en contra del *M. leprae*.

RÉSUMÉ

Les protéines de surface de *Mycobacterium smegmatis* ont été iodées par la méthode à la lactoperoxydase. L'électrophorèse sur gel de dodecyl sulfate sodique de polyacrylamide a permis de mettre en évidence deux protéines principales de surface chez *M. smegmatis* marqués par des radioisotopes. L'une et l'autre de ces protéines de surface ont été libérées de *M. smegmatis* au moyen d'un détergent non ionique, le Triton X-100. Le principal constituant de surface pouvait être digéré par la pronase et ne contenait aucun hydrate de carbone pouvant être détecté. On a trouvé que le second constituant marqué aux radioisotopes avait un poids moléculaire faible, résistait à la digestion par la pronase, et pouvait être coloré par la méthode l'acide périodique de Schiff pour les hydrates de carbone. Les protéines de surface marquées aux radioisotopes et solubilisées par le Triton X-100, étaient antigéniques lorsqu'on utilisait une épreuve de précipitation radioimmune. Lorsque *M. smegmatis*

marqué en surface était mélangé avec du tissu hépatique d'armadillos, et ensuite séparée de ce tissu au moyen d'une méthode employée précédemment par le programme immunologie de la lèpre de l'Organisation Mondiale de la Santé pour la purification de *M. leprae*, on a observé que les protéines de surface de *M. smegmatis* étaient, jusqu'à 50%, soit libérées soit détruites. De plus, une vingtaine d'autres protéines distinctes ont été libérées de *M. smegmatis* après traitement par le Triton X-100. On peut observer des pertes semblables de protéines de *M. leprae* lorsqu'on utilise ce procédé pour la purification du bacille. Les techniques de séparation qui font appel à des surfactants et aux traitements enzymatiques devraient être évaluées soigneusement, car les protéines qui peuvent disparaître au cours de ces manipulations pourraient être en relation avec les réponses immunitaires de l'homme à *M. leprae*.

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