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M. leprae Binds to a 28–30-kDa Phosphorylated Glycoprotein of Rat Peripheral Nerve[1]

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Many pathogenic bacteria specifically recognize and bind to the carbohydrate moieties of the host cell surface ([8]). One of the hallmarks of leprosy is the infection of peripheral nerves by *Mycobacterium leprae* ([9, 10]). Recent studies in nasal epithelial cells have shown that *M. leprae* bind to the extracellular matrix protein fibronectin (FN). This, in turn, binds to integrin FN receptors which help in the internalization of the bacilli ([2, 18]). However, little is understood about the *M. leprae*-peripheral nerve protein interaction.

Protein phosphorylation is a post-translational modification of proteins important in signal transduction and is especially prominent in nerve tissues ([4, 5]). Recent studies have shown the involvement of phosphorylation events in the binding of bacteria to the host cells ([1, 16, 17]).

Earlier studies from our laboratory have shown that purified *M. leprae* could inhibit the phosphorylation of a 28–30-kDa protein of the rat peripheral nerve and of a 25-kDa protein of the human peripheral nerve ([12]). One of the mechanisms proposed for the observation was an interaction between *M. leprae* and the phosphorylated proteins.

In continuation of this observation, we report here that *M. leprae* binds to the following phosphorylated proteins from the rat peripheral nerve: a) a major 28–30-kDa protein and b) a few minor proteins in the molecular weight range of 45–55 kDa. Further, we have characterized the 28–30-kDa protein as a glycoprotein.

MATERIALS AND METHODS

Tris hydroxymethyl aminomethane (Tris), benzamidine hydrochloride, phenyl methyl sulphonyl fluoride (PMSF) and sodium orthovanadate were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A., and gamma ^{32}P ATP was obtained from the Bhaba Atomic Research Centre, Bombay, India. Other reagents used were of analytical grade.

Rat peripheral nerve. Rats (3 months old) were sacrificed by cervical dislocation. The sciatic nerves and brain were dissected and further processed at 4°C. The tissue was minced and homogenized in Tris HCl buffer pH 7.6 (10 ml/g tissue), containing 0.001% (w/v) benzamidine hydrochloride, 200 μM PMSF, 0.1 mM sodium orthovanadate and 0.1% (v/v) Triton X-100. The tissue homogenate was centrifuged at 10,000 × g × 30 min in a Sorval refrigerated centrifuge. The supernatant was used for the phosphorylation of proteins. Protein was determined according to Lowry, *et al.* ([14]). The rat brain was isolated and processed as described for the peripheral nerve.

Protein phosphorylation. Protein phosphorylation conditions were as standardized earlier in our laboratory ([13]). The reaction mixture contained 20 mM Tris-HCl buffer pH 7.5, 20 mM magnesium acetate and gamma ^{32}P-labeled ATP (10×10^7 cpm) and 150 μg of protein in 150 μl of final volume. The reaction was carried out at 30°C for 10 min, at the end of which it was either subjected to SDS-gel electrophoresis or cooled on ice and used for binding studies.

Bacilli. *M. leprae* were isolated from the skin biopsies of borderline lepromatous leprosy patients and from the foot pads of

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inoculated mice and stored at −20°C prior to use as described earlier ([12]). The final bacilli pellet was resuspended in 1 ml of 10 mM phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA). *Escherichia coli* was obtained from cultures provided by the Wellcome Research Laboratory of this Institution. *M. bovis* was obtained from The Tuberculosis Research Centre, Madras, India. *E. coli* and *M. bovis* were heated at 100°C for 2 min prior to use. Lepromin H was prepared as described earlier ([15]). The storage of *M. leprae* and its treatment with NaOH, and heating of *E. coli* and *M. bovis* caused the inactivation of any protein kinases and protein phosphatases in these organisms. *M. leprae*, lepromin H, *M. bovis* and *E. coli*, each of 10^7 bacilli, washed and suspended in PBS containing 0.1% BSA, were used for binding studies.

Binding assay. The binding assay methodology was based upon earlier studies ([6, 22]). The bacilli suspended in 100–400 μl PBS containing 0.1% BSA was added to the ^{32}p phosphorylated nerve proteins. This was incubated at 4°C with gentle shaking for 2 hr. To the suspension, 50 volumes of buffer containing 145 mM NaCl, 10 mM Tris HCl, pH 7.2, and 0.1% (v/v) Triton X-100 was added and microfuged for 1 min. Under these conditions the bacilli were sedimented. The pellet was resuspended in PBS and sedimented by centrifugation. This step was repeated 7–10 times at the end of which 100 μl of SDS dissociation buffer [186 mM Tris HCl buffer, pH 6.8, 6% (v/v) beta-mercaptoethanol, 20% (v/v) glycerol, 6% (w/v) sodium dodecyl sulfate and 0.001% (w/v) bromophenol blue] was added to the pellet and heated at 100°C for 3 min. Similar incubations were carried out using *M. bovis*, *E. coli* and lepromin H. SDS-gel electrophoresis was carried out on 12.5% gels according to Laemmli ([11]). Further processing and autoradiography of the dried gels were carried out as mentioned earlier ([13]).

Concanavalin A-Sepharose column chromatography of phosphorylated proteins. Rat peripheral nerve proteins were phosphorylated as mentioned earlier. The phosphorylated proteins (400 μg) were applied on a column of concanavalin A-Sepharose (0.5 × 3.0 cm) and the glycoproteins were eluted by alpha methyl glucoside. The details of the procedure are as mentioned earlier ([13]).

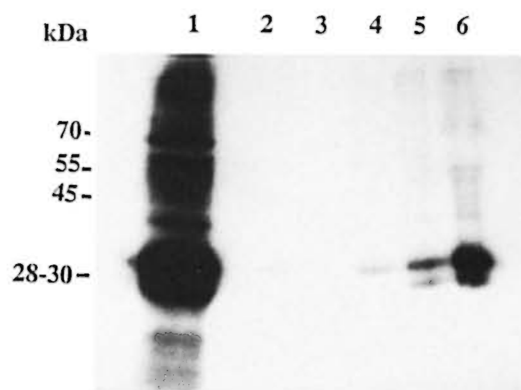

FIG. 1. Autoradiogram showing phosphorylated proteins: Lane 1 shows ^{32}P-phosphorylated peripheral nerve protein (150 μg) (^{32}P-PNP). The same amount (150 μg) of ^{32}P-PNP was used for the binding assay with each of the bacilli. Lanes 2–5 show the ^{32}P-PNP which are bound to the bacteria (10^7 bacilli used in each case) after the binding assay. The binding assay was carried out as described in Materials and Methods. Bacilli used were: Lane 2 = lepromin H; Lane 3 = nil; Lane 4 = *E. coli*; Lane 5 = *M. bovis*; Lane 6 = *M. leprae*.

Periodic acid-Schiff staining for glycoprotein. Staining for the glycoproteins on gel after SDS-gel electrophoresis of proteins was done using the periodate-Schiff reagent according to Segrest and Jackson ([19]).

RESULTS

An autoradiogram after SDS-gel electrophoresis of the reaction mixtures in which rat peripheral nerve extract was incubated with gamma ^{32}P ATP under the assay conditions showed ^{32}P-labeled proteins of molecular weights ranging from 14–200 kDa (Fig. 1, lane 1).

The ^{32}P-labeled rat nerve proteins were incubated with *M. leprae*, and the protein bound to the bacteria was isolated by centrifugation as described in Materials and Methods. This *M. leprae*-protein complex was subjected to SDS-gel electrophoresis and autoradiography. The autoradiogram showed a major 28–30-kDa protein and minor bands in the range of 45–55 kDa (Fig. 1, lane 6), indicating that these proteins could bind to *M. leprae*. Similar experiments with *M. bovis* (Fig. 1, lane 5) showed relatively reduced levels of the 28–30-kDa

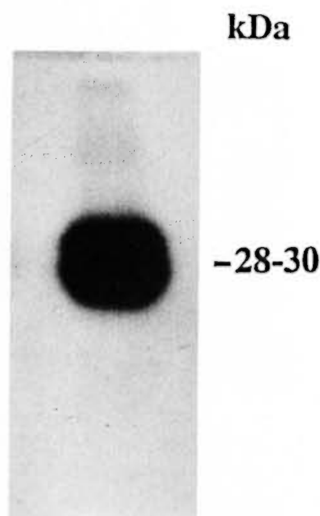

FIG. 2 Autoradiogram showing ^{32}P-phosphorylated 28–30-kDa protein eluted from the concanavalin A-Sepharose column, subjected to SDS-gel electrophoresis (12.5% gel).

protein and the other minor bands. *E. coli* (Fig. 1, lane 4) and lepromin H (Fig. 1, lane 2) showed only insignificant levels of the bound 28–30-kDa protein. There were no ^{32}P-labeled proteins in the control tube in which the binding assay was carried out with the ^{32}P-labeled proteins in the absence of bacilli (Fig. 1, lane 3).

Proteins of *M. leprae, E. coli* and *M. bovis* did not undergo any phosphorylation as observed in separate control experiments.

When the ^{32}P-labeled proteins of the rat peripheral nerve were passed through the concanavalin A-Sepharose column, the 28–30-kDa protein was found to bind to the column. It could be eluted with alpha methyl glucoside (Fig. 2). The 28–30-kDa protein also stained positive with periodate-Schiff's staining on gel (result not shown).

Similar experiments were carried out with the rat brain phosphorylated proteins. None of the bacteria used in this study showed any binding to the brain ^{32}P-phosphorylated proteins (results not shown).

DISCUSSION

As mentioned earlier, *M. leprae* bind to the extracellular matrix protein FN in the nasal mucosal cells and this, in turn, binds to integrin FN receptors which help internalization of the bacilli ([2, 18]). However, this mechanism does not explain the specificity of the bacilli for the nerve tissue.

To explain the tissue specificity of certain pathogenic bacilli, another type of host pathogen interaction has been described recently wherein pathogenic bacteria bind to specific mammalian cell membrane protein receptors present on target cells. *Staphylococcus aureus* is found to bind to the 50-kDa protein of endothelial cells ([22]) and *Ureaplasma urealyticum* to the 30-kDa, 17-kDa, 16-kDa and 14-kDa proteins of human epithelial cells ([21]). These studies have shown that the host cell membrane protein receptors play a major role in tissue specificity of bacterial adhesion and invasion.

The 28–30-kDa protein is the major phosphorylated glycoprotein of the rat peripheral nerve eluted from the concanavalin A-Sepharose chromatography column by alpha methyl glucoside. The molecular weight and the phosphorylatable and glycoprotein nature of the protein indicate that it could be the P_0 protein of the peripheral nerve ([7]). P_0 protein is the major myelin protein of the peripheral nerve and has a significant role to play as a molecular Velcro protein between myelin membranes ([3, 20]). Phosphorylated brain proteins did not show binding to *M. leprae*, suggesting that *M. leprae* has specificity to peripheral nerve tissue protein.

The binding of *M. leprae* to the major 28–30-kDa protein and a few minor 45–55-kDa proteins was significantly higher compared to that of *M. bovis* and *E. coli*. This may be due to the specificity of *M. leprae* in binding the protein. As observed in our earlier work, the decreased phosphorylation of the 28–30-kDa protein in the presence of *M. leprae* could be due to the binding of *M. leprae* to this protein ([12]).

The present work is focused on the phosphorylated host tissue protein binding to *M. leprae*. Further experiments are needed to identify the 28–30-kDa phosphoprotein and the nature of the 45–55-kDa phosphorylated proteins which bind to *M. leprae*, and to know whether any nonphosphorylated proteins of peripheral nerve could bind to *M. leprae*.

In conclusion, the present investigation shows that intact *M. leprae* bind to a major 28–30-kDa protein and a few minor 45–55-kDa proteins of the rat peripheral nerve

phosphorylated *in vitro*. This *M. leprae*-protein interaction could have a role in pathogenesis. The 28–30-kDa protein is the major phosphorylatable glycoprotein of the rat peripheral nerve that resembles the P_0 protein of the peripheral nerve in its characteristics.

SUMMARY

To understand *Mycobacterium leprae*-peripheral nerve interaction, we have investigated the binding of *M. leprae* to rat peripheral nerve proteins in an *in vitro* model using ^{32}P-phosphorylated proteins of the peripheral nerve. Intact *M. leprae* binds to a major phosphorylated protein of 28–30 kDa and, to a minor extent, to a few proteins of molecular weight 45–55 kDa. This binding was more specific for *M. leprae* since only insignificant binding was observed with other bacteria, such as *M. bovis* or *Escherichia coli*. *M. leprae* did not show binding to several phosphorylated proteins of the rat brain. The 28–30-kDa binding protein of the rat peripheral nerve was found to be a glycoprotein by concanavalin A-Sepharose column chromatography.

RESUMEN

Para entender la interacción entre *Mycobacterium leprae*, y los nervios periféricos, investigamos el enlazamiento *in vitro* de *M. leprae* a proteínas fosforiladas (^{32}P) de nervios periféricos de la rata. El microorganismo intacto se enlaza a una proteína fosforilada de 28–30 kD y en manor grado a algunas proteínas de 45–55 kD. El enlazamiento fue relativamente específico para *M. leprae* puesto que sólo se observó un enlazamiento insignificante con otras bacterias como *M. bovis* o *Escherichia coli*, *M. leprae* no mostró enlazamiento con varias proteínas fosforiladas del cerebro de la rata. La proteína enlazadora de 28–30 kD de los nervios periféricos fue una glicoproteína que se pudo separar por cromatografía en columna de sefarosa-concanavalina A.

RÉSUMÉ

Afin de comprendre l'interaction *Mycobacterium leprae*-nerf périphérique, nous avons analysé les liaisons du *M. leprae* aux protéines des nerfs périphériques du rat dans un modèle *in vitro* utilisant des protéines des nerfs périphériques phosphorylées au ^{32}P. Le *M. leprae* intact se lie à une protéine majeure phosphorylée de 28–30 kDa et, dans une moindre mesure, à quelques protéines de poids moléculaire 45–55 kDa. Cette liaison était plus spécifique pour *M. leprae*, puisque seules des liaisons non significatives ont été observées avec d'autres bactéries, telles que *M. bovis* ou *Escherichia coli*. *M. Leprae* n'a pas montré qu'il se liait à différentes protéines phosphorylées du cerveau de rat. On a trouvé par chromatographie en colonne sur concanavaline A-Sépharose que la protéine de liaison de 28–20 kDa du nerf périphérique de rat était une glycoprotéine.

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