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Binding of α2-laminins by pathogenic and non-pathogenic mycobacteria and adherence to Schwann cells

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The ability of *Mycobacterium leprae* to specifically bind α2-laminins of Schwann cells has been described recently as being an important property of the leprosy bacillus, which could explain the neural tropism of *M. leprae*. Therefore, the extent of the expression of α2-laminin-binding properties among mycobacteria was investigated. In an ELISA-based assay, all three species of *Mycobacterium* tested (*M. tuberculosis*, *M. chelonae* and *M. smegmatis*) expressed laminin-binding capacity, suggesting that the ability to bind α2-laminins is conserved within the genus *Mycobacterium*. This report also demonstrated that not only *M. leprae* but all the mycobacterial species tested readily interacted with the ST88-14 cells, a human schwannoma cell line, and that the addition of soluble α2-laminins significantly increased their adherence to these cells. These results failed to demonstrate the presence in *M. leprae* of a unique system based on α2-laminins for adherence to Schwann cells.

Introduction

Unlike other pathogenic mycobacteria that reside inside cells of the mononuclear phagocyte system, *Mycobacterium leprae*, the aetiological agent of leprosy, preferentially infects the Schwann cells of the non-myelinated peripheral nerves leading to a variety of deformities seen in leprosy [1]. Microbial adhesion to host tissue constitutes the first critical event in the pathogenesis of most infections. It often occurs by way of specific interactions between microbial components (adhesins) and extracellular matrix molecules [2]. Recently, the neural tropism of *M. leprae* has been attributed to the specific binding of the leprosy bacillus to laminin-2 [3], a laminin isoform present in peripheral nerve, striated muscle and placenta [4]. Laminins are large heterotrimeric glycoproteins specifically located in the basement membranes. They are made up of three chains of classes α, β and γ and exist in numerous trimeric isoforms in different tissues. In peripheral nerve, laminin-2, comprised of the $\alpha 2$, $\beta 1$ and $\gamma 1$ chains, is present in the base membrane that covers Schwann cells. The same study [4] also demonstrated that *M. leprae* binds to the globular (G) domain of the $\alpha 2$ chain, indicating that laminin may serve as a bridge between the bacteria and the native laminin receptors on Schwann cells.

In the present study, the extent of the expression of $\alpha 2$-laminin-binding properties, as well as the capacity of $\alpha 2$-laminins to mediate the attachment of mycobacteria other than *M. leprae* to human Schwann cells, were investigated.

Materials and methods

Reagents

Human merosin (a mixture of the $\alpha 2$-containing laminins: isoforms –2 and 4), laminin-1 and fibronectin were purchased from Gibco BRL (Gaithersburg, MD, USA). Collagen IV and rabbit anti-laminin antibody were purchased from Sigma. Monoclonal antibody (MAb) specific for merosin was purchased from Gibco BRL. $\alpha 2$-Laminins were labelled with biotin with the FluoReporter Mini-biotin XX protein labelling kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions.

Mycobacteria

M. leprae was purified from livers and spleens of infected armadillo as described previously [5], or

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harvested from footpads of HSD nu/nu mice (donated by James Krahenbuhl, Baton Rouge, LA, USA). *M. tuberculosis* H37 Rv ATCC 27294 was cultivated at 37°C in glycerol-alanine-salts medium [6]. *M. bovis* BCG Pasteur 1173P2 WHO, *M. smegmatis* ATCC 19420 and *M. chelonae* NCTC 946 were grown at 37°C in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with Tween 80 0.05%, glycerol 2% and ADC supplement. The bacteria were harvested in mid-log phase and kept frozen (−80°C) in divided small volumes until use.

Human schwannoma cells

The ST88-14 schwanomma cell line was isolated from a patient with neurofibromatosis type I [7] and kindly donated by Professor Jonathan Fletcher (Harvard University, Boston, MA, USA). The cell line was maintained in RPMI medium (Gibco BRL, Rockville, MD, USA) supplemented with fetal calf serum 15%, penicillin 100 U/ml, streptomycin 100 μg/ml and 2 mM L-glutamine in an incubator at 37°C in air with CO_2 5%.

Binding of soluble α2-laminins to mycobacteria in microtitration plates

To monitor the binding of soluble laminin to mycobacteria, bacterial suspensions in 0.1 M carbonate buffer, pH 9.6, at saturating concentrations (100 μl) were used to coat the wells of polystyrene microplates (Corning, New York, NY, USA). Plates were incubated for 4 h at 37°C followed by another 4-h period at 4°C. The wells were then washed with phosphate-buffered saline (PBS) (10 mM phosphate buffer, pH 7.2, 0.15 M NaCl) and blocked overnight with 200 μl of PBS-bovine serum albumin 2% (BSA). After washing with PBS/Tween 20 0.05% (PBS/T), 100 μl of biotinylated α2-laminins (5 μg/ml) were added to the wells which were incubated at 37°C for 3 h. The wells were rinsed with PBS/T and incubated with streptavidin-peroxidase (Pierce, Rockford, IL, USA) 0.5 μg/ml. Peroxidase activity was determined with H_2O_2 and *o*-phenylenediamine (OPD). The reaction was stopped with HCl and the result was read at 490 nm in a TitertekPlus microplate reader (ICN Biomedicals, Costa Mesa, CA, USA). Laminin was omitted from control wells and specific laminin binding was determined by subtracting the absorbance resulting from non-specific binding detected in the control wells. Also, control wells coated with BSA were included in all binding assays. Competition experiments with α2-laminins were performed in the presence of 20-fold excess of unlabelled α2-laminins, laminin-1, fibronectin and collagen IV. *M. leprae*-coated wells were incubated simultaneously with biotinylated α2-laminins and competitors.

Immunohistochemistry

ST88-14 cells (7×10^4cells/ml) were plated in 24-well plates containing glass coverslips. After incubation for

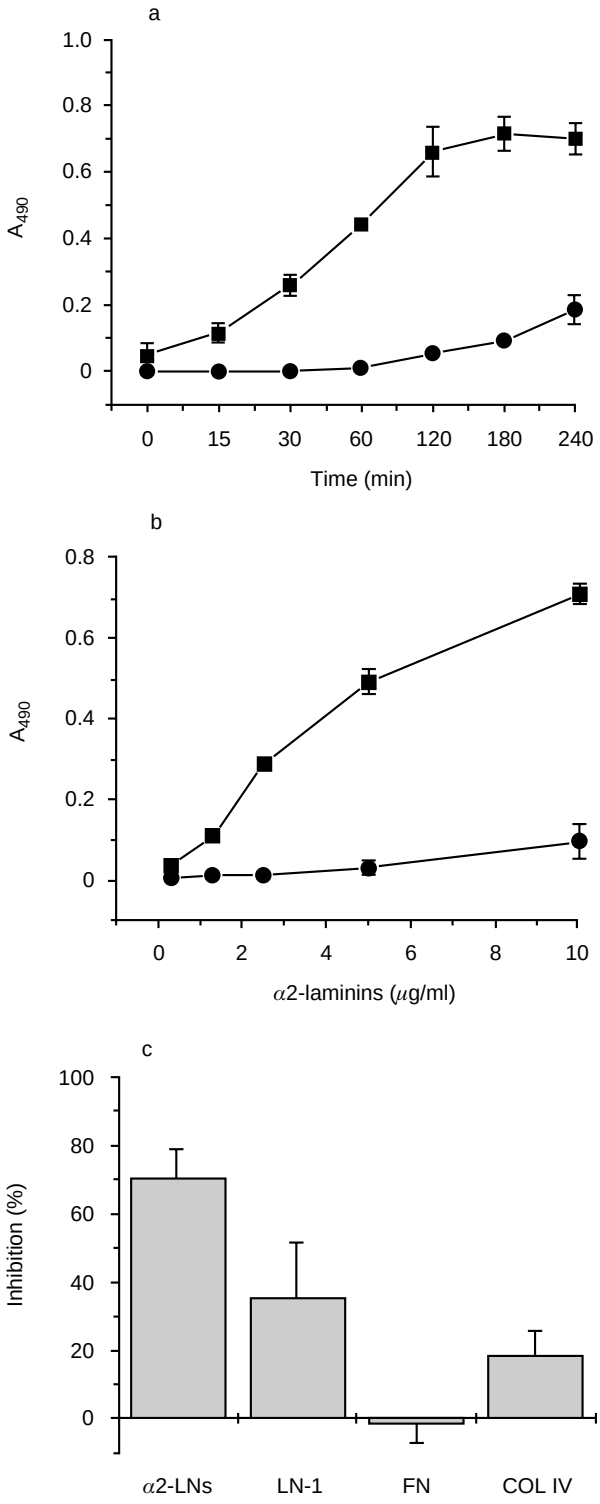

Fig. 1. Characteristics of the interaction of *M. leprae* with α2-laminins. (**a**) Time course study of the binding of soluble α2-laminins to *M. leprae*. (**b**) Binding of increasing concentrations of soluble α2-laminins to *M. leprae*. (**c**) Specificity of binding of α2-laminins to *M. leprae*. Microtitration wells coated with *M. leprae* (■) and BSA (●) were incubated with biotinylated α2-laminins. The inhibitory compound was simultaneously added at 20-fold higher concentration to biotinylated α2-laminins to microtitration wells coated with *M. leprae*. Data are expressed as percentages of inhibition, where binding to bacteria incubated in the absence of potential inhibitors was set as 0% inhibition. Data represents the mean and SEM of five experiments done in triplicate. α2-LN, α2-laminins; LN-1, laminin-1; FN, fibronectin; COL, collagen.

24 h, the culture was washed three times with 25 mM Tris-HCl, (pH 7.4, 5 mM CaC, 0.15 M NaCl (TBS). Immunohistochemical studies were performed to examine the presence of α2-laminins on the surface of ST88-14 cells by the streptavidin-biotin-peroxidase complex procedure (ABC). In brief, coverslips were incubated with rabbit anti-laminins or with anti-α2-laminins specific MAb (1 in 100 in PBS) overnight at 4°C followed, respectively, by a goat anti-rabbit and anti-mouse IgG biotinylated antibody (Dakopatts, Copenhagen, Denmark). Controls for the ABC procedure were performed omitting the anti-laminin antibodies. The cells were analysed by light microscopy and photomicrographs were taken with a Nikon Microphot system.

Bacterial adherence assays

ST88-14 cells (7×10^4cells/ml) were plated in 24-well plates containing glass coverslips. After incubation for 24 h, the culture was washed three times with a solution of 25 mM Tris-HCl (pH 7.4) 5 mM $CaCl_2$, 0.15 M NaCl (TBS) and blocked with BSA 1% in TBS for 1 h at 37°C. FITC-labelled mycobacteria were prepared as described earlier [8] and were added to the cells at a multiplicity of infection of 50:1. After incubation for 1 h at 37°C, cells were washed at least six times with TBS, stained with ethidium bromide 50 μg/ml for 10 min and fixed briefly with cold methanol. The number of bacteria attached to 100 cells was counted by fluorescent microscopy (Optiphot-2 Nikon, Japan).

Results and discussion

Expression of laminin-binding properties by mycobacterial species

To investigate the interaction of mycobacteria with α2-laminins, a simple assay was developed in which the bacteria were immobilised in wells of microtitration plates and the binding of soluble biotinylated α2-laminins was assessed with the streptavidin-peroxidase complex. The characteristics of the interaction of *M. leprae* with α2-laminins were initially analysed in this assay. *M. leprae* isolated from two different sources (infected armadillo and nude mouse) displayed identical laminin-binding capacities in this assay and were indiscriminately used in this study (data not shown). Soluble biotinylated α2-laminins bound to *M. leprae* in a time-dependent manner (Fig. 1a) and in a concentration-dependent way (Fig. 1b). This reaction reached a plateau in 2 h (Fig. 1a). Continuation of incubation for up to 4 h did not significantly increase the amount of biotinylated laminins bound to *M. leprae*. In subsequent experiments, the incubations were routinely for 3 h to ensure maximal binding. Binding of biotinylated α2-laminins to *M. leprae* was dependent on the bacterial density originally coated on the well and reached a maximum at bacterial saturation concentration of 20 μg/ml (data not shown). The specificity of α2-laminin binding to *M. leprae* was further studied in competitive experiments in which unlabelled α2-laminins, laminin-1, fibronectin and collagen IV were added to microtitration wells in 20-fold excess to labelled α2-laminins. Fig. 1c shows that unlabelled α2-laminins strongly inhibited the binding of biotinylated α2-laminins to the bacteria. Although laminin-1

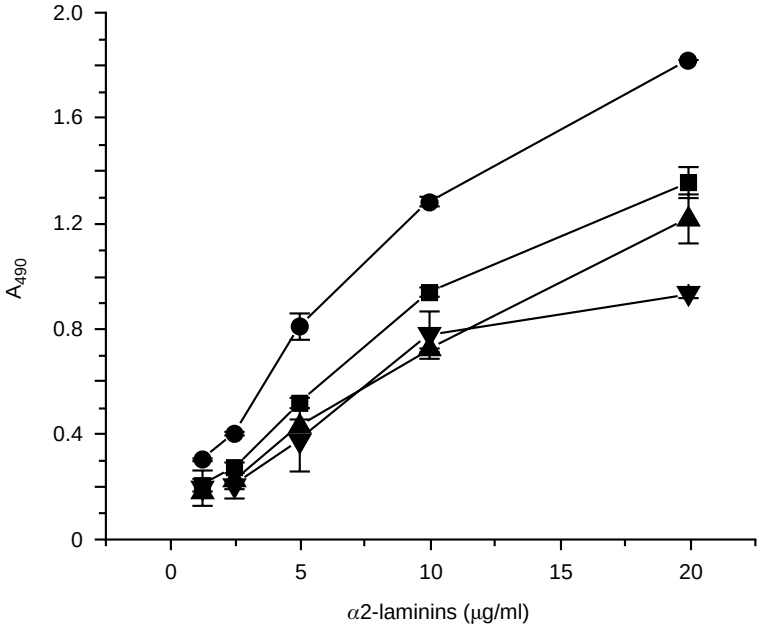

Fig. 2. Binding of mycobacterial species to α2-laminins. Microtitration wells coated with saturated amounts of *M. leprae*(■), *M. chelonae* (▼), *M. tuberculosis* (●) and *M. smegmatis* (▲) were incubated with different concentrations of biotinylated α2-laminins. Binding is expressed in absorbancy units at 490 nm. Data represent the mean and SD of a typical experiment done in triplicate. Five experiments were performed with similar results.

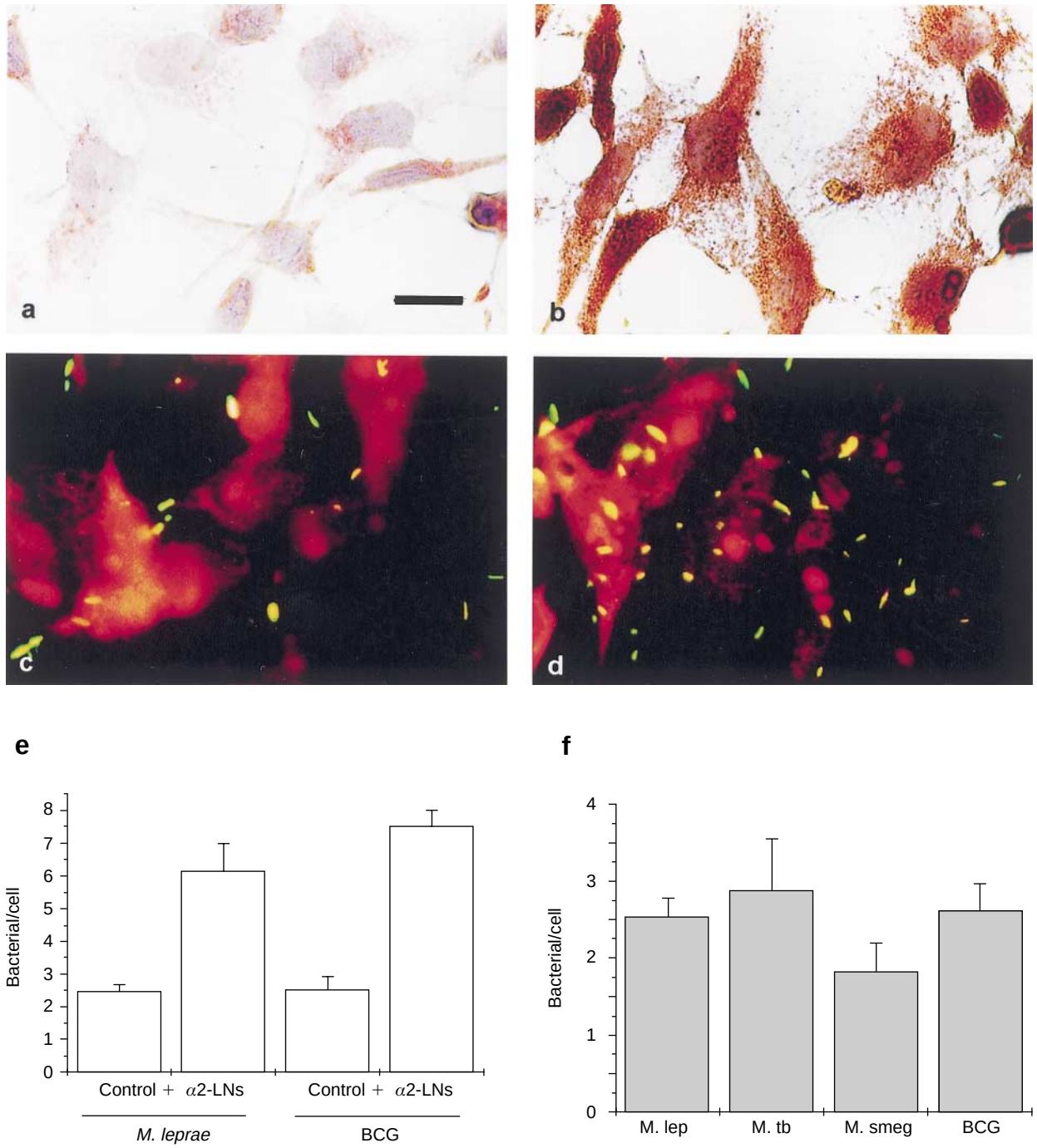

Fig. 3. (**a, b**) ST88-14 cells express laminins. ST88-14 cells were cultivated on coverslips and immunostained with a rabbit anti-laminins antibody (**b**). In the control (**a**), cells were stained omitting the primary antibody. (**c, d, e**) Pre-treatment of mycobacteria with α2-laminins increased their adherence to Schwann cells. Cells were incubated with FITC-labelled *M. leprae* (**c**) or with FITC-labelled *M. leprae* pre-treated with soluble α2-laminins (**d**). After incubation for 1 h, unbound bacteria were removed by washing and cells were stained with ethidium bromide and analysed by fluorescence microscopy. In (**e**) results are expressed as the average number of bacteria per cell. Data represent the mean and SEM of five experiments in duplicate. (**f**) Binding of mycobacterial species to Schwann cells. Cells were incubated with FITC-labelled mycobacteria for 1 h and the average number of bacteria attached per cell was determined by fluorescence microscopy. Data represent the mean and SEM of seven experiments done in duplicate. Bar, 10 μm.

substantially inhibited binding, it did not do so as effectively as α2-laminins. On the other hand, fibronectin and collagen IV were ineffective as inhibitors. The results obtained in this assay are in agreement with those described previously in which a different bacterial binding assay that consisted of determining the number of bacteria bound to extracellular matrix proteins immobilised on Terasaki plates was used [4].

Comparative assays were then performed to determine

whether the α2-laminin-binding property displayed by *M. leprae* was shared with other species of *Mycobacterium*. *M. tuberculosis*, which does not cause nerve damage, was shown to bind α2-laminins. Moreover, *M. chelonae* and *M. smegmatis*, two avirulent species of *Mycobacterium*, displayed laminin-binding capacities (Fig. 2). These results demonstrated that the ability to bind α2-laminins was present in all mycobacteria tested, including both pathogenic and opportunist species, suggesting that it is conserved within the *Mycobacterium* genus.

Adherence of mycobacterial species to human schwannoma cells via α2-laminins

The interaction of *M. leprae* with the human schwannoma cell line ST88-14 was also investigated. Initial immunohistochemical analysis of the ST88-14 cells with an anti-laminin antibody demonstrated intense immunoreactivity in the perinuclear region and in the processes of all cells, as shown previously [9] (Fig. 3a and b). When immunostained with a specific MAb for the laminin α2-chain, ST88-14 demonstrated the same staining pattern, indicating that these cells express abundant α2-laminins (data not shown). The interaction of FITC-labelled *M. leprae* with these cells was then investigated and an average of 2.6 (SEM 0.22) bacteria/cell was observed after incubation for 1 h at 37°C (Fig. 3c and f). Similar results were obtained when *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis* were used in identical adherence assays (Fig. 3f). These results reinforce those of previous studies indicating that mycobacterial invasion of cultured Schwann cells is not specific to *M. leprae*. In one of these studies [10], the capacity of Schwann cells present in human nerve teased-fibre preparations to engulf both *M. lepraemurium* and carbon particles besides *M. leprae* was demonstrated. In another study [11], seven species of mycobacteria, including *M. tuberculosis*, *Mycobacterium w*, *M. smegmatis* and *M. vaccae* were shown to be phagocytosed by rat schwannoma cell line 33B. In the same study, all mycobacteria tested were also phagocytosed by rat primary Schwann cells.

The influence of soluble α2-laminins in this interaction was then tested. By pre-treating *M. leprae* with soluble α2-laminins (100 μg/ml), its adherence to ST88-14 cells was significantly increased to 6.5 (SEM 0.9) bacteria/cell (Fig. 3d and 3e). The same phenomenon was observed with BCG (Fig. 3e) as well as with *M. tuberculosis* and *M. smegmatis* (data not shown), suggesting that in this in-vitro system α2-laminins are acting as mediators in attachment of both *M. leprae* and BCG to Schwann cells. The enhanced binding effect of α2-laminins observed in these experiments might be explained by the capacity of laminin molecules to self-assemble and form polymers [12].

In summary, this study demonstrated that α2-laminins bind to pathogenic or non-pathogenic mycobacteria and then mediate their adherence to Schwann cells. Whether *M. leprae*, but not other species of *Mycobacterium*, is able to bind the G domain of the laminin α2-chain [13] needs further investigation. However, a recent report [14] as well as our own observations (Marques *et al*., unpublished results) have shown that a *M. leprae* histone-like protein, a highly conserved protein found in other species of mycobacteria, is capable of binding to the G domain of laminin-2 and may function as the adhesin for mycobacterial attachment to Schwann cells. In this context, the results presented here indicate that the ability of *M. leprae* to adhere to Schwann cells *via* α2-laminins should not be considered the reason for the uniqueness of *M. leprae* among pathogenic mycobacteria in its ability to invade Schwann cells inside the host as previously suggested [4]. In fact, tissue tropism is mediated by a complex set of host and microbe-derived factors, most of which are still unknown. In the case of *M. leprae*, migration to the nerve probably involves multiple steps in which both specific and non-specific factors are required.

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