

# Multiple Endothelial Membrane Proteins Bind *M. leprae*

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## Abstract

Morphologic evidence has suggested that endothelial cells (EC) may be the gateway through which *M. leprae* enter peripheral nerve. Studies *in vitro* have demonstrated that uptake of *M. leprae* by EC is time- and dose-related. Experiments have therefore been undertaken to identify the EC membrane proteins capable of binding *M. leprae*.

Cytoplasmic membranes from 12x10<sup>6</sup> EC grown *in vitro* were solubilized and their proteins conjugated to biotin. *M. leprae* (2x10<sup>9</sup>) were allowed to bind these biotinylated proteins for 4 hr at 4°C. The bacterial pellet was washed to remove unbound proteins; bound proteins were separated by SDS-PAGE and electro-transferred to PVDF membranes. Biotinylated EC proteins were visualized by staining with an avidin-alkaline phosphatase conjugate.

Biotinylated EC proteins bound to *M. leprae* were separated into several distinct bands, 7 of which have been consistently identified in different experiments. In these preliminary experiments, the smaller molecules (29, 32, 47, and 54 kDa) have yielded discrete single bands on 8% and 10% gels; the larger molecules have appeared more diffuse, with bands at 59-63, 125-130, and 175-185 kDa.

These studies suggest that EC are capable of binding *M. leprae* using multiple surface proteins. Although these probably include proteins already used by other cell types to *M. leprae*, they may also include binding proteins unique to EC.

## Introduction

Evidence from an animal model suggests that endothelial cells (EC) may be the 'gatekeepers' enabling *M. leprae* to enter nerves via their blood vessels (1,2). One mechanism by which this could occur would involve receptors on EC membranes binding to molecules on *M. leprae*.

Functionally distinct properties of neural endothelium, especially with respect to leukocyte adhesion, have been described in experimental models of neuritis and encephalitis (3).

Known receptors for specific binding of *M. leprae* include complement receptors on macrophages (4, 5), fibronectin (6), and the  $\alpha_2$  isoform of laminin in the Schwann cell basement membrane (7, 8). Recent studies have also demonstrated that *M. leprae* binds to several phosphorylated proteins, including a human 25 kDa protein. (9, 10).

As illustrated in Fig. 1, *M. leprae* would bind to EC in the vessel lumen and pass through the EC into the endoneurial compartment, where they are bound by Schwann cells. We have therefore initiated studies to demonstrate and identify EC membrane proteins that bind *M. leprae* *in vitro*.

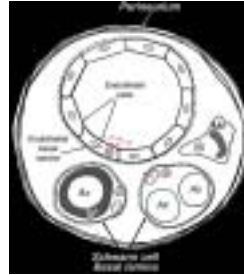


Figure 1. Diagram of a small nerve fascicle containing a blood vessel and both myelinated and non-myelinated Schwann cells.

If *M. leprae* enter nerves via their blood supply, as proposed, then in order to reach the Schwann cell they must first adhere to and pass through (or between) endothelial cells and cross their basal lamina. Once in the interstitium of the fascicle, bacilli may be bound and ingested by resident macrophages (M $\phi$ ), or they may bind to the basal lamina of myelinated (M) or non-myelinated Schwann cells which then ingest them. (From ref. 3) (Not illustrated is the possibility that infected circulating mononuclear phagocytes adhere to and migrate between endothelial cells, carrying *M. leprae* into the endoneurium).

## Materials & Methods

Human umbilical vein endothelial cells (EC) were cultured and membranes from approximately 12 x 10<sup>6</sup> EC were isolated by differential centrifugation. These EC membranes were solubilized to extract their proteins according to the protocol summarized in the schematic below.

*M. leprae* were freshly obtained from infected nude mouse footpads and were used promptly, without freezing or irradiating.

### Protocol

Isolate EC membranes, dissolve proteins with triton X-100 in borate buffer

Biotinylate & dialyze ('B-EC proteins')

Incubate B-EC proteins with *M. leprae* x 4h, 4°C

Wash to remove non-bound B-EC proteins

SDS-PAGE of *M. leprae* with bound proteins

Transfer to PVDF membrane

Stain with streptavidin-alkaline phosphatase (SA-ap)

### Result:

EC membrane proteins (biotinylated) are stained;  
*M. leprae* proteins (not biotinylated) do not stain

## Results

Studies of solubilized, biotinylated EC proteins that bind to *M. leprae* *in vitro* have consistently demonstrated at least 7 bands by SA-ap staining of PVDF blots (Figure 2).

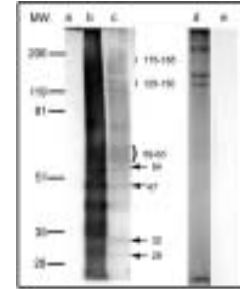


Figure 2. Demonstration of EC proteins binding *M. leprae* by SA-ap staining.

EC proteins were solubilized, biotinylated, and incubated with *M. leprae* as described. After washing, *M. leprae* and bound proteins were separated by SDS-PAGE on an 8% gel, and electro-transferred to PVDF membrane. Biotinylated EC proteins (B-EC) were demonstrated on the membrane by SA-ap staining.

- a: Control, non-biotinylated EC proteins: no proteins were stained on PVDF blots, although bands were seen on the gel by Coomassie blue staining (not shown).
- b: The fraction of B-EC proteins not bound to *M. leprae* consistently yielded strong staining throughout on PVDF blots, with only a few discernable bands.
- c: **B-EC bound to *M. leprae* were separated into several distinct bands on PVDF blots, 7 of which have been consistently identified** (arrows and brackets).
- d: Gel separation of control samples with *M. leprae* alone revealed several bands (i.e., *M. leprae* proteins) by Coomassie blue staining.
- e: None of the *M. leprae* proteins (in d) were stained with SA-ap on PVDF blots.

## Conclusions & Discussion

At least 7 EC membrane protein bands were associated with *M. leprae* binding. These may each represent different individual proteins, although some may be fragments of larger proteins resulting from denaturation and reduction in SDS-PAGE.

Identification of these proteins remains to be determined. Some are expected to be proteins used by other cells to bind *M. leprae*, while others may be uniquely used by endothelial cells.

Both common and unique binding proteins may play important roles in the entry of *M. leprae* into nerves through blood vessels, as proposed in the model described in Figure 3.

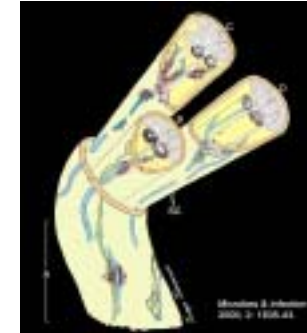


Figure 3. A cutaneous nerve with three fascicles is represented here to illustrate the proposed steps in the pathogenesis of infection of peripheral nerves by *M. leprae*.

(A) Initially, colonization of the epineurium (e) occurs when bacilli (red) localize in cells in and around blood vessels (blue). It is possible that this is enhanced by drainage of bacilli through the lymphatics (green) which are intertwined with the blood vessels of the epineurium (lymphatics are here illustrated only at the lower end of the drawing). The resulting accumulation of bacilli within and around endothelial cells greatly increases the likelihood that bacilli will be available for circulation through the endoneurial vessels which branch off of the epineurial ones.

(B) Entry of *M. leprae* into the endoneurial compartment proceeds along blood vessels from foci on and within the perineurium (p), extending through it into the interior of the nerve (See also Fig 2). The mechanisms responsible for entry into the interstitial space of the endoneurium remain to be determined. Once inside, however, bacilli are available for phagocytosis by Schwann cells (SC), represented here with concentric layers of myelin surrounding axons. Although these initial events in the localization and entry of *M. leprae* into peripheral nerve are postulated to be unrelated to specific immune function, the subsequent pathogenesis of neuritis in leprosy probably depends in large part on the patient's immune response to *M. leprae*.

(C) If no effective immune response develops (e.g., lepromatous leprosy) bacilli proliferate within macrophages and Schwann cells. This results in perineurial inflammation and thickening ('proliferation'), and an increasing bacterial load both in the epineurium and in the endoneurium. Since *M. leprae* is an indolent, well-adapted intracellular pathogen, however, axons are not badly damaged for an appreciable length of time, and a variable degree of nerve function is preserved until late in the course of the disease.

(D) If effective cellular immunity and delayed hypersensitivity do develop (e.g., tuberculoid leprosy), a granulomatous response follows at sites of infection in epineurial and endoneurial vessels and Schwann cells. This immunologically-elicited inflammation eliminates nearly all of the bacilli in the epi- and perineurium, and also stimulates perineurial fibrosis and thickening. However, *M. leprae* which have already been ingested by Schwann cells may be relatively protected from immunologically mediated destruction and are able to maintain a persistent infection in these cells for a long time. This is where most bacilli are found in diagnostic biopsies of tuberculoid lesions.

Granulomatous inflammation is also potentially injurious to adjacent tissue. In *M. leprae*-infected nerves, this includes injury to axons in the vicinity of the granulomas, resulting in impaired nerve function. (From Ref. 3)

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