学位論文

「The unique tropism of *Mycobacterium leprae* to the nasal epithelial cells can be explained by the mammalian cell entry protein 1A (*Mycobacterium leprae* は mammalian cell entry 1A 蛋白を用いて鼻粘膜上皮細胞へ侵入する)」

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著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

The unique tropism of *Mycobacterium leprae* to the nasal epithelial cells can be explained by the mammalian cell entry protein 1A

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背景と目的

ハンセン病は *Mycobacterium leprae* (*M. leprae*) の感染により皮膚や末梢神経が侵される慢性の抗酸菌感染症である。現在ハンセン病の新規患者数は、先進国では激減したが、世界では年間 20 万人を超えており、Neglected Tropical Diseases, NTDs の一つとして、依然として公衆衛生上の大きな問題となっている。しかし、らい菌の人工培養法が確立されていないことが研究の妨げとなり、らい菌の感染様式は解明されないままに隔離政策を経て現在に至った。Mammalian cell entry 1 A (mce1A) 蛋白は結核菌の mce1A 領域にコードされる蛋白で、結核菌の上皮系細胞への侵入やマクロファージ内における生存, 増殖への関与が報告されている。結核菌 mce1A 領域と相同性の高い領域が、らい菌にも存在する事が明らかとなり、リコンビナント蛋白を用いた研究から本領域が、らい菌の上皮系細胞への侵入にも関わる重要な領域である事が報告された。今回、らい菌の上皮系細胞への侵入に関わる mce1A 領域の active sequence を明らかにする事を目的に検討を行った。

方法および結果

最初に、mce1A 蛋白が、native 蛋白としてらい菌に発現していることを免疫電子顕微鏡法にて確認した。

大腸菌を用いて作製したらい菌 mce1A 領域(1326bp、442aa)の N 末及び C 末をトランケートした各種組換え蛋白(r-lep37KDa、r-lep 27KDa)をラテックスビーズにコートし、HeLa 細胞への侵入活性を電子顕微鏡を用いて検討した結果、316bp ～ 921bp(202aa) までの r-lep 27KDa でも侵入活性が維持された。次に、316～921bp の領域を 316～531bp、532～753bp、754～921bp に 3 分割し、各領域を AIDA ベクターに組み込み大腸菌の菌体表面に表出させた。各組換え大腸菌を単層培養した HeLa 細胞及び RPMI2650 細胞に加え、菌の細胞内への侵入をコロニーカウント法と電子顕微鏡を用いて観察した。その結果、316～531bp の領域でのみ侵入が観察された。

次に、これまでの研究結果から想定された侵入活性領域 (316～531bp の領域) を

316-387bp（106-129aa）、388-453bp（130-151aa）、454-486bp（152-162aa）、および487-531bp（163-177aa）に 4 分割して、夫々の領域に対する高度免疫血清を作製し、316bp-531bp（72aa 領域）を外膜表示した組換え大腸菌を用いて侵入抑制効果を検討した結果、106-129 アミノ酸と 130-151 アミノ酸および 163-177 アミノ酸を免疫原に作製した高度免疫血清（抗 InvXa 抗体と抗 InvXb 抗体および抗 InvXd 抗体）に侵入抑制効果が認められた。

考察

今回の結果から、*M. leprae* の鼻粘膜上皮細胞への侵入に関与する active sequence は *mce1A* 領域の 316～531bp 間に存在することが明らかとなった。結核菌 Mce1A 蛋白のヒト上皮細胞への侵入に係わる最も重要な領域は、Inv3 領域と呼ばれ、130～152 アミノ酸に位置することが同定されている。結核菌の Inv3 領域は、らい菌では、388-453bp （InvXb）に相当するが、その配列は、N 末端部分から数えて 1～22 番目のアミノ酸はほぼ同一であるが、1～3、5、8、9、13 番目のアミノ酸がらい菌と結核菌では異なっており、抑制試験の結果からもらい菌 Mce1A 蛋白のヒト上皮細胞への侵入に係わる最も重要な領域は、結核菌のそれとは異なった領域である事が示唆された。

M. leprae は、蛋白をコードする遺伝子がわずか 1,604（*Mycobacterium tuberculosis*（*M. tuberculosis*）では 3,959）である一方、偽遺伝子は 1,116（*M. tuberculosis* では 6）存在していることから *M. leprae* では多くの蛋白が結核菌と比して不活化されている。Mce1A 蛋白は、結核菌と同様に native 蛋白として菌体表面に発現していることから、本蛋白は *M. leprae* の鼻粘膜上皮細胞への侵入に関与する重要な蛋白の一つであり、ハンセン病の感染予防においてワクチンのターゲットとなりうる重要な領域であると考えられる。

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1. Introduction

Hansen's disease is a chronic infection with acid fast bacillus where skin and peripheral nerves are damaged by the infection with *Mycobacterium leprae* (*M. leprae*). Although the number of Hansen's disease cases has drastically decreased in developed countries, worldwide, the number of new cases of Hansen's disease has only dipped below 200,000 per year. Hansen's disease is one of the Neglected Tropical Disease (NTDs) and is still a major problem against public health[1].

Hansen's disease can be broadly divided into tuberculoid leprosy (T type) and lepromatous leprosy (L type), depending on the host immune response to *M. leprae*[2]. Tuberculoid leprosy triggers predominantly cellular immunity response, and is also called paucibacillary, because very few are detected at the focus of infection or nasal mucosal membrane. On the other hand, lepromatous leprosy triggers predominantly humoral immunity, and is also called multibacillary, because it is detected in a large amount at the focus of infection and, in particular, from nasal mucosal membrane. Nasal discharge from lepromatous leprosy patients, therefore, is considered as the main source of the infection[3]. Infection of Hansen's disease has conventionally been considered to occur through close skin contact or through wounds, but recently another infection mode, in which *M. leprae* in the aerosol from nasal discharge of lepromatous leprosy patients invades into the upper respiratory tract and nasal mucosal membrane to cause infection, has come to be recognized[3-10]. However, the invasion mechanism in this infection mode has not been extensively studied yet.

M. leprae cannot be artificially cultured. One possible reason for this is the presence of a large number of pseudogenes. *M. leprae* has various enzyme-coding genes that are replaced with pseudogenes, and therefore has only a minimum metabolic activity and multiplies in macrophages and Schwann cells. Invasion mechanism of *M. leprae* into Schwan cells have been studied

by Rambukkana, et al., in details. The study revealed that the binding of *M. leprae* to dystroglycan of Schwann cells in the presence of laminin-2 requires phenolic glycolipid PGL-1 and 21KDa protein (ML1683) on the bacteria surface to enter the Schwann cells[11-14].

To infect Schwann cells, *M. leprae* has to invade the epithelial cells first. The mechanism of *M. leprae* invasion into the epithelial cells, however, has not been elucidated yet. Meanwhile, gene regions involved in the invasion of *Mycobacterium tuberculosis (M. tuberculosis)* into epithelial cells are already known[15, 16]. Casali et al. reported that, using adhesin involved in diffuse adherence (AIDA) method, the region coded for by 316 - 531 bp of *M. tuberculosis mce1A* region (Rv 0169; 198534 - 199898 bp, 1365 bp) is expressed on the surface of *E. coli* as a polypeptide chain, thereby imparting the *E. coli* with the ability to invade HeLa cells, that the invasion activity is inhibited by the monoclonal antibody (Ab) that recognizes the continuous peptide of InvIII region (388 - 453 bp)[17-19]. It became clear that *M. leprae* includes a region (ML2589, 1326 bp) highly homologous to Mce1A protein of *M. tuberculosis*. Sato et al. reported that a recombinant protein, a 37 kDa protein encoded by 73 - 921 bp, which is the Mce1A region excluding the signal sequence, was found to have an invasion activity into epithelial cells[20]. However, the active sequence involved in the invasion by *M. leprae* into epithelial cells has not been identified. The present study was conducted to identify the active sequence in the Mce1A region. In this study, the N-terminus and C-terminus truncated proteins expressed on the *E. coli*, where *E. coli* with specific regions are expressed thereon by the AIDA method, and hyperimmune antisera against the invasion region are used to investigate the invasion activity into epithelial cells.

2. Material and Methods

2-1 Bacterial strains and plasmid

The genomic DNA used in the study was isolated from *M. leprae* strain Thai

53, which was maintained at Leprosy Research Centre, National Institute of Infectious Diseases, Japan, as previously described[21,22]. The pQE30 plasmid and *E. coli* M15 (pREP4) were purchased from Qiagen (Valencia, CA). The pQE30 plasmid was used as expression vector. *E. coli* M15 (pREP4) was used as a host for the vector, as recommended by the manufacturer.

The pMK90 plasmid and *E. coli* UT4400 were obtained from Dr. Riley (University of California at Berkeley, California, USA).

2-2 Construction of vector

In Sanger Center *M. leprae* strain TN complete genome sequence, *mce1A* gene is a 1326 bp putative ORF located between positions 3092446 and 3093771 (NCBI-GeneID: 910890). The *mce1A* DNA sequence of strain Thai 53 was identical to that of strain TN. It was subcloned into pQE30 vector in a truncated reading frame. The 603 bp ORF deleted at 5' and 3' ends of *mce1A* gene is located between positions 316 and 921 (Fig 1). This sequence was amplified by polymerase chain reaction (PCR) directly from the genomic DNA of *M. leprae* strain Thai 53 with oligonucleotide primers designed to introduce *Sac*I and *Hin*dIII endonuclease restriction sites at the ends. The amplified products were ligated into the pQE30 vector linearized with *Sac*I and *Hin*dIII. The use of pQE30 vector allowed the plasmid to express the Mce1A product with a polyhistidine (6 x His) tag at the N-terminus (r-Mce1A). The resultant plasmid was cloned into *E. coli* M15 (pREP4) by electroporation (Gene Pulser II, Bio-Rad, Hercules, CA), according to the manufacturer's instructions.

Plasmid pMK90 is an ampicillin-resistant pBR322 derivative that expresses a recombinant AIDA protein under the control of its own promotor[22]. The AIDA coding sequence has been altered to remove the native passenger; it consists of a 49-amino-acid signal peptide. A 78-amino-acid linker with the entire 440 amino acid barrel core is incorporated between *Xma*1 and *Xba*1 of the multiple cloning site. A 216 bp

DNA fragment encoding *invX* (*M. leprae* positions 3092761 - 3092976 bp), 222 bp DNA fragment encoding *invY* (*M. leprae* positions 3092977 - 3093198 bp), 168 bp DNA fragment encoding *invZ* (*M. leprae* positions 3093199 - 3093366 bp) was amplified by PCR from a plasmid containing *mce1A* and cloned into pMK90, generating UT4400/pMK*invX*, UT4400/pMK*invY*, and UT4400/pMK*invZ*. The correct insert was confirmed by sequencing. The amino acid sequence of InvX is VNADIKATTVFGGKYVSLTTPEHPSQKRLTPQTVIDARSVTTEINTLFQTITLIAEKVDPIKLNLTLSAA AQ (316 - 531 bp), the amino acid sequence of InvY is SLAGLGERFGQSIVNGNSVLDDVNSQLPQARHDIQQLASLGDTYANSASDFFDFLNNSIVTSRTINQQQK DLDQ (532 - 753 bp), and the amino acid sequence of InvZ is VLLAAVGFGNTGADIFSRSGPYLARGAADLVPTAQLLDTYSPAIFCTLRNYHDIEP (754 - 921 bp) (Fig 1).

2-3 Protein expression and purification

Recombinant protein was expressed and purified according to manufacture' s instruction. Briefly, *E. coli* M15 [pREP4] containing pQE30/*mce1A* plasmid was grown overnight in 10=ml superbroth containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. A 500 μl aliquot of bacterial suspension was pelleted, resuspended in 30 ml of superbroth and incubated at 37 °C for 1 - 2 h until OD_{600} = 0.6. Then isopropyl β-D-thiogalactoside was added to final concentration 1 mM and incubated for 3 h at 37 °C. The induced and uninduced r-*E. coli* strains were analyzed by SDS-polyacryamide gel electrophoresis (SDS-PAGE). The newly expressed protein formed an inclusion body in the r-*E. coli* host. The inclusion body was therefore purified under denaturing conditions according to the instructions of the expression vector' s respective manufactures. The 6 x His tag Mce1A solubilized with lysis buffer (6 M guanidine, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0) was bound to a Ni-NTA resin column equilibrated with lysis buffer, and was eluted by elusion buffer (6 M guanidine, 10 mM Tris-HCl, 100 mM

NaH_2PO_4, 20–250 mM imidazole, pH 6.3). The eluted protein were subsequently refolded with 1 mM dithiothreitol (Sigma, St. Louis, MO, USA) and 0.1 mM phenylmethylsulfonyl fluoride (Sigma) by dialysis, gradually removing guanidine. The r-Mce1A was finally purified and refolded as a soluble protein. The purified r-Mce1A protein (2 μg) is migrated using SDS-PAGE, and a single band was confirmed with Coomasie brilliant blue R-250 staining.

2-4 Immunoelectron microscopy

The Ab against the Mce1A protein was prepared in BALB/c mice (a 45 kDa recombinant Mce1A protein prepared previously using *E. coli* was used as the immunogen). The r-45kDa- Mce1A protein was used for Ab production because it was most abundantly expressed in the *E. coli* host that we used. The r-45kDa-Mce1A protein was mixed with Titer Max Gold (AdipoGen Life Sciences, Liestal, Switzerland) of the same amount. Approximately 100 μg of the protein was administered subcutaneously at five sites in four 7-weeks-old BALB/c mice, followed by two booster injections of 100 μg each 2 and 4 weeks after the first injection. Regarding the specificity of the Ab, Western blot analysis revealed that it reacts with the whole-cell lysate of *M. leprae* strain Thai 53 and this was used for experiment.

A bacterial pellet (containing $\approx 10^7$ organisms) of *M. leprae* strain Thai 53 that had been multiplied in footpads of athymic nude mice, was fixed in 3% glutaraldehyde in phosphate buffer saline (PBS) pH 7.6 for 24 h, washed five times in PBS and then exposed at 4 °C for 16 h to a 1:1000 dilution of the mice Ab raised against Mce1A. The suspension was then washed and incubated at 4 °C for 16 h with colloidal gold suspension containing 5 nm gold particles (1.9×10^{13} particles/ml) conjugated to anti-mouse IgG goat Ab (Amersham/GE Health Care Life Science, Tokyo, Japan). The cells were washed again five times in PBS, stained with 0.1% uranyl acetate in water and examined with a HITACHI model H-15 electron microscope.

2-5 Cell Culture

HeLa cells and RPMI2650 cells were purchased from America Type Culture Collection (ATCC, Manassas, VA). HeLa cells (ATCC CCL-2) were maintained with Dulbecco's modified Eagle's media (DMEM; Invitrogen, Carlsbad, CA) supplemented with 50 μg/ml gentamicin (GM) and 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS). RPMI2650 human epithelial nasal septal cell line (ATCC CCL30) was grown in Eagle's minimum essential medium (EMEM; Invitrogen, Carlsbad, CA) supplemented with 50 μg/ml GM and 10% FBS. Cells were maintained in culture and for the assay, were detached from the plastic by using 0.25% Trypsin-EDTA (1×) with phenol red (Gibco, Grand Island, NY, USA) at 37 °C. The cells were then centrifuged at 280 × g for 7 min at 4 °C, counted in Neubauer hemocytometer, and plated into tissue culture well or flask at 37 °C in a 5% CO_2 atmosphere.

2-6 Cell uptake assay of protein-coated latex beads by electron microscopy

A 30 μl of stock suspensions of 1.1 μm diameter polystyrene latex beads, containing 5 × 10^8 beads/ml (Sigma), were mixed in 150 μl of PBS containing 50 μg/ml of each set of protein and incubated for 16 h at 37 °C. After incubation, the samples were centrifuged at 7000 × g and resuspended in 750 μl of PBS. A 500-μl sample of this suspension was added to a near-confluent cultured cell monolayer grown in a 25-cm^2 flask containing 7 ml of appropriate media for cultured cells. The cells were incubated for 5 h at 37 °C in a CO_2 incubator, washed four times with PBS and one time with 0.1 M cacodylate phosphate buffer (pH 7.6), and then collected with cell-scraper (Becton Dickinson, Japan). The collected cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate phosphate buffer (pH 7.6) at 4 °C overnight, post-fixed with 1% osmium tetroxide in PBS, dehydrated through graded ethanol solutions and embedded in Spurr's low-viscosity embedding media. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1200EX (JEOL, Tokyo, Japan) transmission electron microscope. Coated

beads with bovine serum albumin (BSA) fraction V (Boehringer Mannheim, GmbH, Germany) were used as negative controls.

2-7 Immunofluorescence microscopy

E. coli (UT4400/pMK*invX*) cells were fixed onto microscope slides with 0.4% paraformaldehyde for 10 min at room temperature, and non-specific binding was blocked by incubation in 1% (w/v) BSA for 30 min. Slides were incubated for 1 h with a 1:200 dilution of a rabbit Ab raised against InvXa, InvXb, InvXc, and InvXd washed, and incubated with 1:1000 dilution of fluorescein isothiocyanate-labeled anti-rabbit Ab (Abcam Plc, Cambridge, UK) for 30 min. Normal rabbit IgG was used for labelling for the negative control. After extensive washing, the coverslips were mounted. Slides were viewed on an Olympus BX51 inverted microscope with an epifluorescence attachment.

2-8 Invasion assay

2-8-1 InvX, InvY, InvZ mediated invasion of *E. coli* into the nasal epithelial cells by electron microscopy

The culture medium of a monolayer culture of 1.15×10^6 HeLa cells and 3×10^7 RPMI2650 cells, was replaced with a culture medium that does not contain antibiotic substances. Then *E. coli* externally expressing by AIDA (UT4400/pMK*invX*, UT4400/pMK*invY*, UT4400/pMK*invZ*) was added at a bacteria to cells ratio of 100:1, and incubated in a CO_2 incubator at 37 °C, for 9 h for HeLa cells and 6 h for RPMI2650 cells. After culturing, the cell surface was washed with PBS, and then harvested using a cell scraper.

Infected cells were prepared for examination by transmission electron microscopy as previously described[16]. Briefly, cells were fixed in 2% glutaraldehyde and stained with osmium tetroxide solution before dehydration through graded ethanol solutions. Cells were embedded in Spurr's low-viscosity embedding medium, ultrathin sections were stained with uranyl acetate and lead citrate. Samples were examined with a JEM-1230 (JEOL)

transmission electron microscope.

2-8-2 GM protection assays

GM protection assays were performed according to the method of Elsinghorst[24]. RPMI2650 cells were seeded at 5×10^5 cells per well directly into 24-well plates and cultured for 24 h until confluent. Cell culture medium was modified to contain no antibiotics. Recombinant *E. coli* cells were added to the monolayer at a multiplicity of infection (MOI) of 10:1 and incubated at 37 °C for 3 h. To enumerate intracellular bacteria, the monolayer was washed five times with PBS and incubated with medium containing 100 μg of GM (Sigma) per ml for 2 h to kill extracellular bacteria and permit the enumeration of intracellular bacteria. The monolayer was again washed five times with PBS and lysed with 0.1% Triton X-100 (Eastman Kodak, Rochester, NY). Serial dilutions of released bacteria were plated for counting. Results shown are the mean values for an experiment performed in triplicate. Each experiment was performed three times using independent cultures, with similar result.

2-9 Inhibition Assay

Anti-InvXa, anti-InvXb, anti-InvXc, and anti-InvXd Abs were added in the amount of 1:200 to *E. coli* that externally express UT4400/pMK*invX* by AIDA adjusted to 1×10^8 CFU/ml. This was allowed to react on a rotating platform at 4 °C overnight to make Ab-treated bacteria. *E. coli* externally expressing proteins by AIDA were allowed to react with IgG from healthy control rabbits in a similar manner as the control, where this was used as the bacteria untreated by Ab. After the medium for RPMI2650 cells, which were monolayer-cultured in a 24-well plates, 5×10^5 cells/well, was replaced with a medium not containing antibiotic agent, the Ab-treated bacteria and untreated bacteria were added at a bacteria to cells ratio of 30:1. After culturing in CO_2 incubator at 37 °C for 3 h, the surface of the cells were

washed with PBS five times, and the medium was replaced with a 100 μg/ml GM-appended DME medium to kill the bacteria outside the cells, followed by additional incubation for 2 h. The surface of cells was washed with PBS, and then 0. 1% Triton X-100-added PBS was added in the amount of 1 ml/well to break the cells and the bacteria inside the cells were harvested. The harvested bacteria suspension liquid was serially diluted 10 times with PBS, and then was applied to Heart Infusion agar medium (Nissui, Tokyo, Japan). This was left overnight at 37 °C, and then the colonies were counted to determine the number of bacteria entered into the cells. The cultured cells were prepared in the amount of 3 wells each, and the average of each well and standard deviation were calculated and the result was presented on a graph.

2-10 Ethics statement

This study was approved by the Institutional Animal Care and Use Committee (Permission number : 2013153) and carried out in accordance with the KITASATO University Animal Experimentation Regulations.

3. Result

3-1 Immunoelectron microscopy of M. leprae

Immunoelectron microscopy was employed to determine whether *M. leprae* expressed the Mce1A protein on the cell surface.

The bacilli expressing Mce1A protein were pretreated with an Ab raised against r-45 kDa Mce1A protein, and were followed by incubation with anti-IgG Ab-conjugated colloidal gold particles (Fig 2). The immunoelectron microscopic study revealed that the native Mce1A protein is expressed on the surface of bacilli. This confirms that the *M. leprae* not only expresses Mce1, but the Mce1 is transported to the cell surface and sufficiently presented such that it can bind the Ab against it.

3-2 HeLa cell uptake assay of protein-coated polystyrene latex microbeads by electron microscopy

The active sequence involved in the invasion into the epithelial cells was investigated in the following manner. The r-lep37kDa protein, which had been prepared in the previous experiment using r-lep45kDa protein as the reference by truncating the C terminus to 308 aa (922 bp), was further truncated to 105 aa (315 bp) from N terminus to provide r-lep27kDa protein where the proteins using were expressed using an *E. coli* expression system (Fig 1). Each of the truncated protein was observed for invasion activity into HeLa cells using an electron microscope. In this observation, images of beads coated with r-lep37kDa protein and beads coated with r-lep27kDa protein invading into the cytoplasm of HeLa cells were captured, but BSA-coated beads, which are the negative control, were not found to invade the cytoplasm (Fig 3). This result suggest that the active sequence is present between 316 - 921 bp, which encodes r-lep27kDa protein.

3-3 InvX, InvY, InvZ mediated invasion of E.coli into the nasal epithelial cells by electron microscopy

The active sequence was further investigated. The 316 - 921 bp region was divided into *invX*; 316 - 531 bp, *invY*: 532 - 753 bp, *invZ*: 754 - 921 bp, and each of the regions was incorporated into AIDA vector to produce a recombinant *E. coli* externally expressing the proteins (Fig 1). The *E. coli* externally expressing the proteins by the AIDA method were observed for invasion activity into HeLa cells and RPMI2650 cells under the electron microscope. *E. coli* expressing InvX (UT4400/pMK*invX*) was found in abundance in the cytoplasm. *E. coli* expressing InvY (UT4400/pMK*invY*), InvZ (UT4400/pMK*invZ*), UT4400, and UT4400/pMK90 were observed present around the cells but not inside the cytoplasm (Fig 4). These results suggest that the active sequence is present in 316 - 531 bp (*invX*).

3-4 InvX, InvY, InvZ mediated invasion of E.coli into the nasal epithelial cells (GM protection assay)

Next, using a GM protection assay, the number of bacteria which entered into RPMI2650 cells was determined in colony forming units (CFU).

To determine uptake of the host *E. coli* cells using a GM protection assay, we assessed the invasive ability of InvX, InvY and InvZ expressing *E. coli* cells showed invasion levels at the 3 h time point.

In RPMI2650 cells, invasive activity of InvX-expressing *E. coli* was significantly higher than that of InvY, InvZ, and negative control (Fig 5). The result was similar to the observations by electron microscopy. Invasion activity into nasal mucosa epithelial cells was successfully imparted to an *E. coli* by externally expressing the InvX region of *M. leprae* on the *E. coli*. The InvX mediates the nasal epithelial cells invasion by non-pathogenic *E. coli*. The InvX region within Mce1A protein is then sufficient for the invasion of *E. coli* into the cells.

3-5 Indirect immunofluorescence staining of InvX expressing E.coli by Abs corresponding to each region of Mce1A

Indirect immunofluorescence was used to determine which regions of Mce1A are sufficient to confer invasive ability to *E. coli*.

In order to examine whether the Abs recognize each of the regions, fluorescence immunostaining was conducted on the Abs. Fluorescence microscopy revealed bacterial surface binding of the InvX Abs by their binding of labelled secondary Abs of fluorescence goat anti-rabbit IgG (Fig 6).

3-6 Inhibitory effects of anti-InvX Abs raised against each set of synthetic peptide corresponding to an InvX divided region on the nasal epithelial cells invasion of InvX expressing *E. coli*

In order to investigate an active site involved in the entry of MceIA protein of *M. leprae* into nasal mucosal cells, we analyzed inhibitory effects of the resultant Abs on the cell uptake of InvX-expressing *E. coli* by the inhibition assay. As shown in CFU analysis, the InvX-expressing *E. coli* pretreated with anti-InvXa Ab, anti-InvXb Ab, and anti-InvXd Ab had significantly lower entry than the IgG control, but there was no significant difference in pretreatment with anti-InvXc Ab and IgG control (Fig 7). These findings suggest that the invasion activity was most suppressed when using Abs to cover the polypeptide chain encoded by 316 - 387 bp and expressed on the surface of *E. coli*.

4. Discussion

A number of studies have been conducted on the infection mode of *M. leprae*. In 1955, Khanolkar et al. reported that *M. leprae* infection of *M. leprae* occurs by normal skin contact[3]. However, in 1963 Weddell et al. revealed that the infection does not occur unless the bacteria is inoculated under the skin[25]. Rees et al. induced immune suppressed mice to inhale an aerosols containing *M. leprae* which successfully infected the mice via upper airway[4]. Following this, Chehl et al. revealed that transnasal infections of *M. leprae* of nude mice was possible[5]. From these studies, it became clear that the infection from aerosol containing *M. leprae* and through the nasal membrane can be established. However, as of today, only limited studies have been conducted regarding molecular mechanisms involved in the invasion.

The Mce region is present in tuberculosis complex such as *M. tuberculosis* and *Mycobacterium bovis*, as well as in atypical mycobacteria such as *Mycobacterium avium* and *Mycobacterium intracellulare*[26]. Chitale et al. revealed that this MceIA protein involved in the invasion into epithelial cells is expressed only in tuberculosis complex[16]. We had found that *M. leprae* has a region highly homologous with to the MceIA region of *M. tuberculosis* (Fig 8), and so far have prepared a recombinant protein

(Mce1A protein) encoded by *mce1A* region (ML2589; 3092446 to 3093771, 1326 bp) of *M. leprae* to investigate invasion activities to epithelial cells[15, 19]. In the present study, we have confirmed that the Mce1A protein was actually expressed, as a native protein, on the surface of *M. leprae*, and prepared a recombinant protein by truncating the N terminus and C terminus of Mce1A region of *M. leprae* to investigate the invasion activity into the epithelial cells. As a result, it was found that invasion activity is maintained even if 105 aa (315 bp) is truncated from N terminus and 308 aa (922 bp) is truncated from C terminus. Next, 316 bp to 921 bp region was divided into 3 parts, and each part was incorporated into an AIDA vector, where each region was externally expressed as a polypeptide chain to investigate whether the ability to invade can be imparted to non-pathogenic *E.* coli. These *E. coli* which externally express the protein by AIDA method were examined for the invasion activity using RPMI2650 cells, where the results indicate that active sequence of *M. leprae* involved in the invasion into nasal mucosa epithelial cells is present in the 316 - 531 bp of *mce1A* region.

The most important region of Mce1A protein involved in the invasion of *M. tuberculosis* into human epithelial cells is called the InvIII cell and this is located between amino acids of position 130 to position 152[19, 27]. The InvIII region of *M. tuberculosis* corresponds to InvXb of *M. leprae*. The sequence of the regions are identical between amino acid of position 1 to position 22 - counted from N terminus of InvXb except that amino acids at positions 1 to 3, 5, 8, 9, 13 are different between *M. leprae* and *M. tuberculosis* (Fig 10). Suppression test results also indicated that the most important region of Mce1A protein of *M. leprae* involved in the invasion into human epithelial cells is different from that of *M. tuberculosis*.

While *M. tuberculosis* has 3,959 protein-encoding genes and only 6 pseudogenes, *M. leprae* has only 1,604 protein-encoding genes but has 1,116 pseudogenes[28], indicating that in *M. leprae*, far more proteins are inactivated as compared to *M. tuberculosis*. Based on this study, it was found that Mce1A protein of *M. leprae* is expressed on the bacteria surface

as a native protein, as it is in *M. tuberculosis*, and that N terminus region of Mce1A protein of *M. leprae*, in addition to the active region of *M. tuberculosis*, is involved in the entry. These findings suggest that Mce1A is one of the most important proteins involved in the invasion of *M. leprae* into nasal mucosa epithelial cells.

Since any effective prevention of infective disease depends on the accurate analysis and understanding of the mode of infection and blocking of the pathway of transmission, we are confident that this paper provides valuable information and inspiration for the prevention of Hansen's disease, including vaccine development and effective control methods targeting the entry protein.

5. Acknowledgement

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6. References

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8. Figures

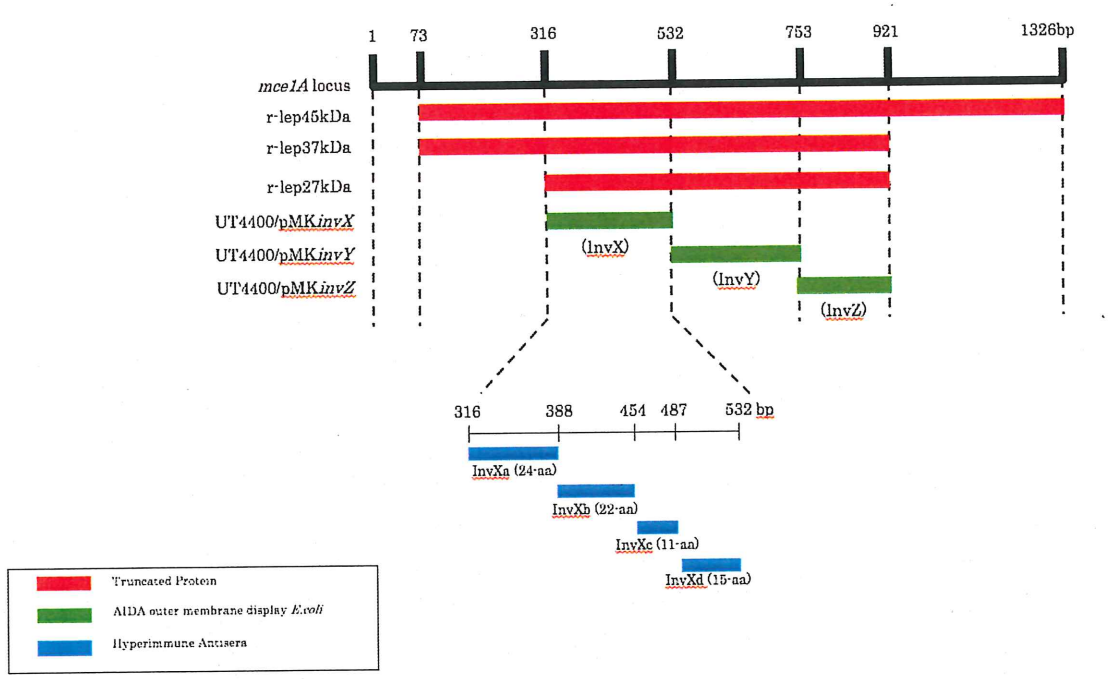

Fig 1. r-Mce1A truncated protein regions expressed on *E. coli*, regions externally expressed using AIDA method, and regions for which hyperimmune antisera is prepared.

Using r-lep45kDa protein encoded in the entire region of Mce1A minus signal sequences (73 - 1326 bp) as a reference, the protein with its C terminus truncated to 921 bp was denoted as r-lep37kDa protein. Where the N terminus of r-lep37kDa is truncated to 315 bp, the resultant construct was denoted as r-lep27kDa protein. Furthermore, 316 - 921 bp region was divided into 316 - 531 bp, 532 - 753 bp, and 754 - 921 bp, and recombinant *E. coli* in which these regions are externally expressed using AIDA method were prepared. InvX region (316 - 531 bp) was further divided into *invXa* (316 - 387 bp), *invXb* (388 - 453 bp), *invXc* (454 - 486 bp), *invXd* (487 - 531 bp). Hyperimmune antisera was prepared by synthesizing peptide for each InvX region (Sigma Custom Peptide Service), adding Keyhole Limpet Hemocyanin (KLH), and inoculating into rabbits.

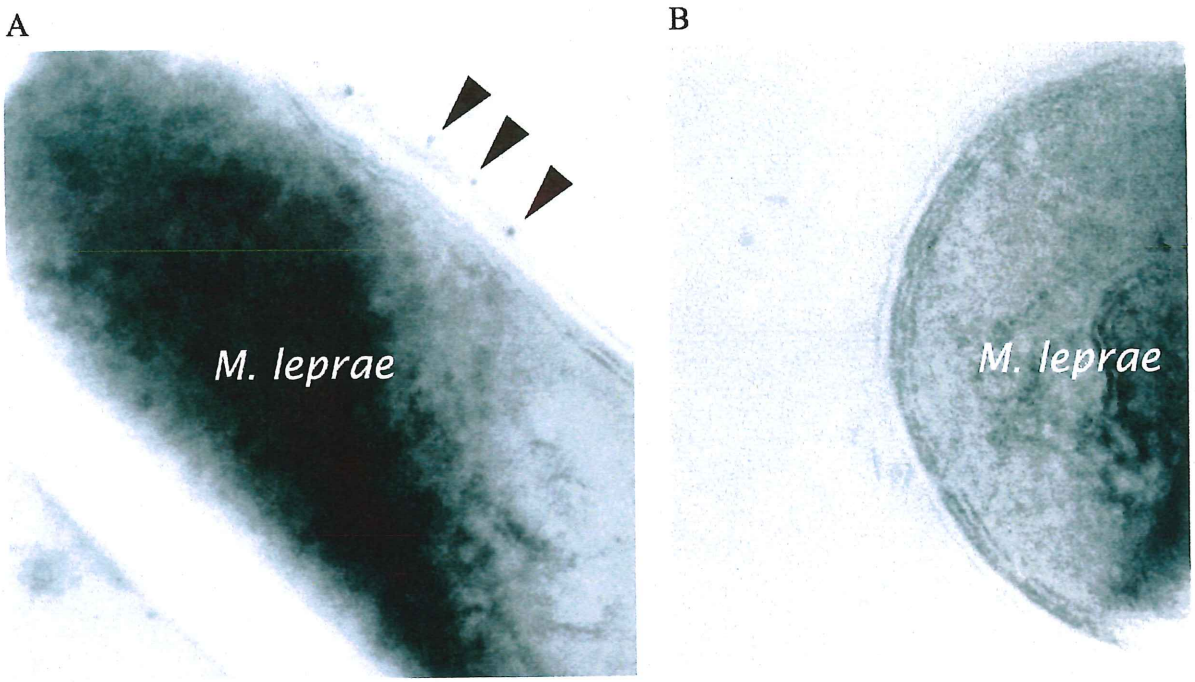

Fig 2. Colloidal gold immunoelectron microscopic analysis of *M. leprae* strain Thai 53.

The bacilli were pretreated with an Ab raised against r-45 kDa Mce1A protein, and were followed by incubation with anti-IgG Ab-conjugated colloidal gold particles. Gold particles are shown decorating the surface of the *M. leprae* bacillus (arrowheads).

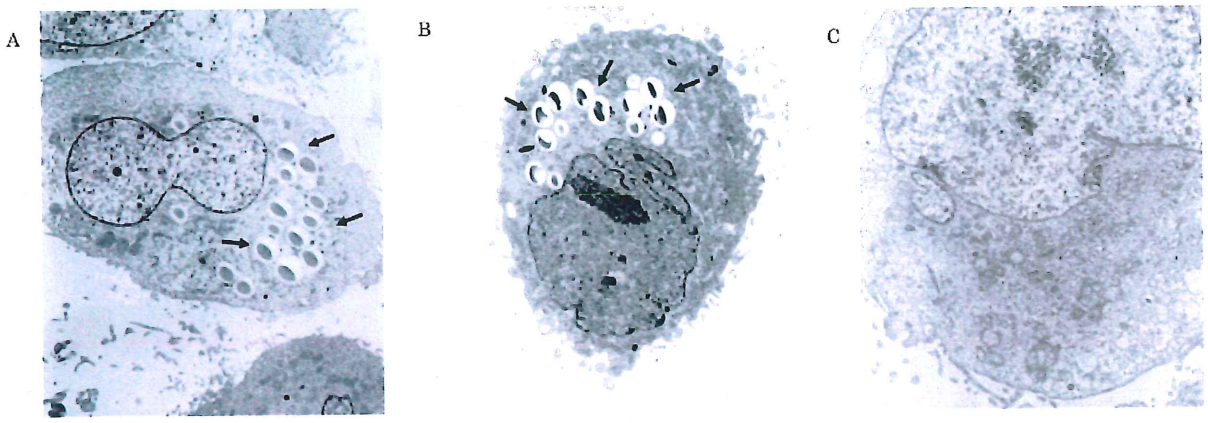

Fig 3. HeLa cells uptake assay of protein-coated polystyrene latex microbeads by electron microscopy.

Monolayer-cultured HeLa cells and the truncated protein-coated beads and BSA-coated beads were allowed to react for 5 h, and the entry of beads into HeLa cells were observed under an electron microscope. As shown in the arrow, (A) r-lep37kDa protein-coated beads and (B) r-lep27kDa protein-coated beads were observed to enter into HeLa cells, but (C) BSA-coated beads were not observed to enter into HeLa cells. Magnification ×3500 (A), ×5000 (B, C)

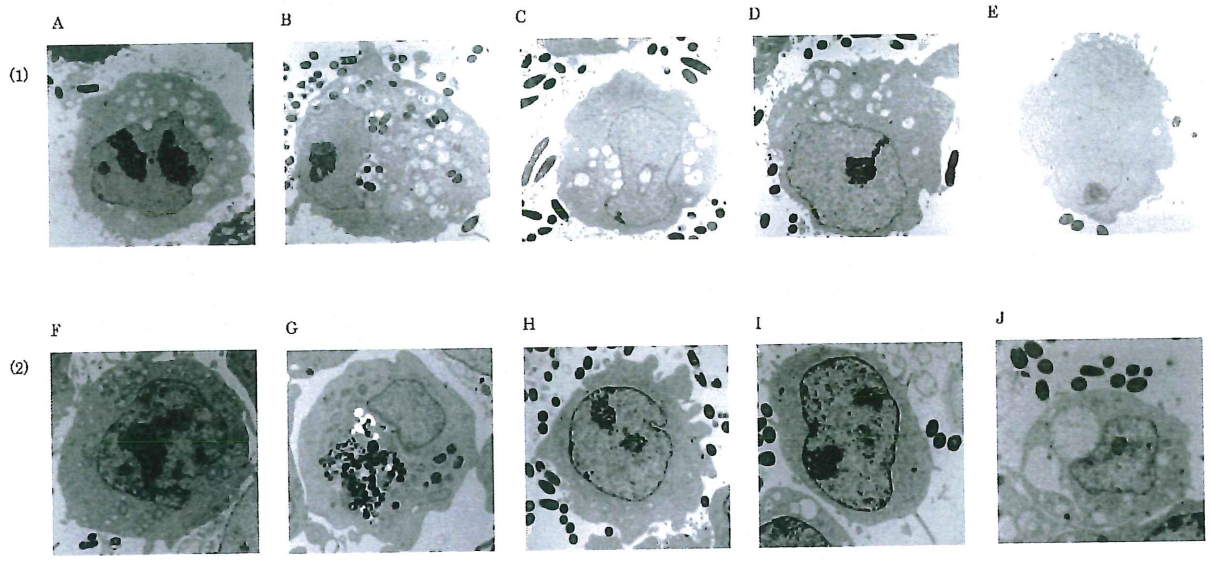

Fig 4. Transmission electron microscopy of InvX, InvY, InvZ mediated invasion of *E. coli* into HeLa and nasal epithelial cells.

UT4400 (A, F), UT4400/pMK*invX* (B, G), UT4400/pMK*invY* (C, H), UT4400/pMK*invZ* (D, I), and UT4400/pMK90 (E, J) were added to monolayer cultured HeLa cells (1) and to RPMI2650 cells (2) at a cell to bacteria ratio of 1:100. They were allowed to react for 9 h and 6 h, respectively, and *E. coli* entry into the cells was observed under an electron microscope. Only UT4400/pMK*invX* (B, G) was observed to invade HeLa cells and RPMI2650 cells. Although UT4400 (A, F), UT4400/pMK*invY* (C, H), UT4400/pMK*invZ* (D, I), and UT4400/pMK90 (E, J) were observed to be present around the cells, no invasion into the cells was observed with them. Magnification ×5000

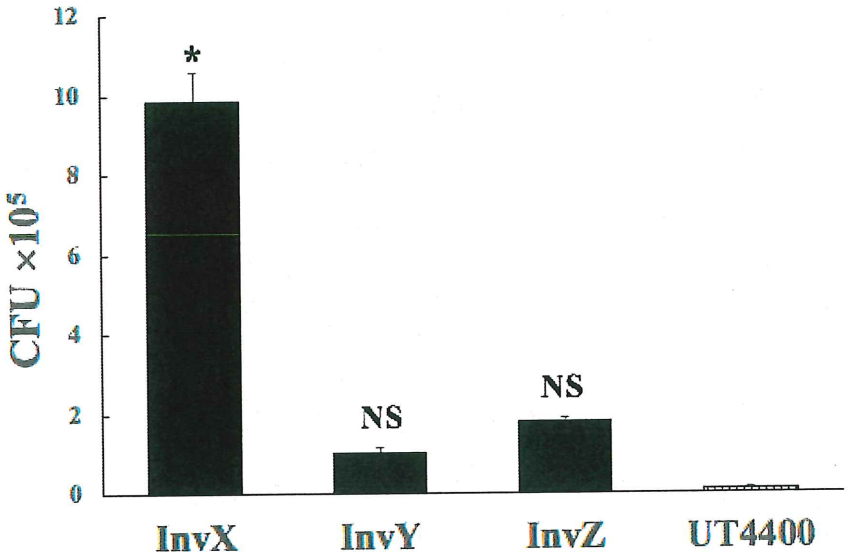

Fig 5. InvX, InvY, InvZ mediated invasion by *E. coli* into the nasal epithelial cells (GM protection assay).

Each of AIDA externally expressing *E. coli* was added to monolayer cultured RPMI2650 cells at a cells to bacteria ratio of 1:10 and the mixture was allowed to react for 3 h. The bacteria outside the cell were subjected to disinfection by GM for 2 h. The bacteria inside the cell were counted with the colony count method, which was presented as CFU. Bars represent the mean ± S.D. of intracellular bacteria as a CFU in a representative experiment performed in triplicate. Asterisks indicate significant difference compared with an IgG control (Scheffe' s multiple comparison test).

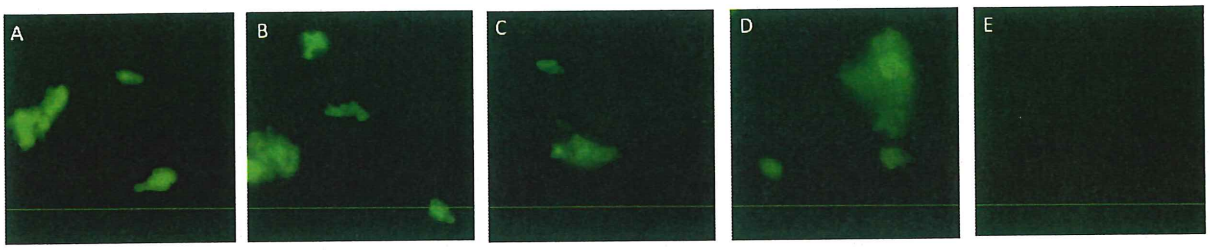

Fig 6. Indirect immunofluorescence staining of InvX expressing *E. coli* by Abs corresponding to each region of InvX.

Visualization of the bacilli reveals bacterial surface binding of the fluorescent goat anti-rabbit IgG to rabbit anti InvX present of the cell surface. (A) InvXa, (B) InvXb, (C) InvXc, (D) InvXd and (E) normal rabbit IgG.

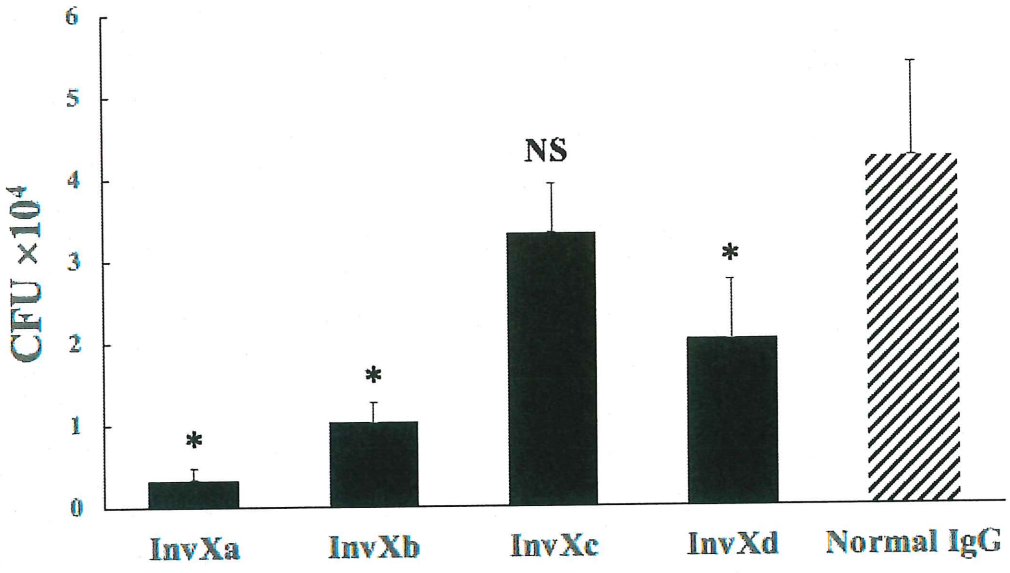

Fig 7. Inhibitory effects on anti-InvX Abs raised against each set of synthetic peptide corresponding to an InvX divided region on the nasal epithelial cells invasion by InvX expressing *E. coli*.

UT4400/pMK*invX E. coli* strain were pretreated overnight with resultant Abs and normal IgG as a control. Each Ab preparation was added to monolayer RPMI2650 cells and incubated for 3 h. After washing, GM was added to eliminate bacteria outside the cells. Bars represent mean ± standard deviation CFU of UT4400/pMK*invX E. coli* treated with Abs. The invasive activity was suppressed by Abs against InvXa, InvXb, and InvXd. Asterisks indicate $p<0.05$, NS: Not significant (Dunnet test). Bars represent the mean ± SD of intracellular bacteria in CFU in a representative experiment performed in triplicate. Asterisks indicate a significant difference compared with an IgG control.

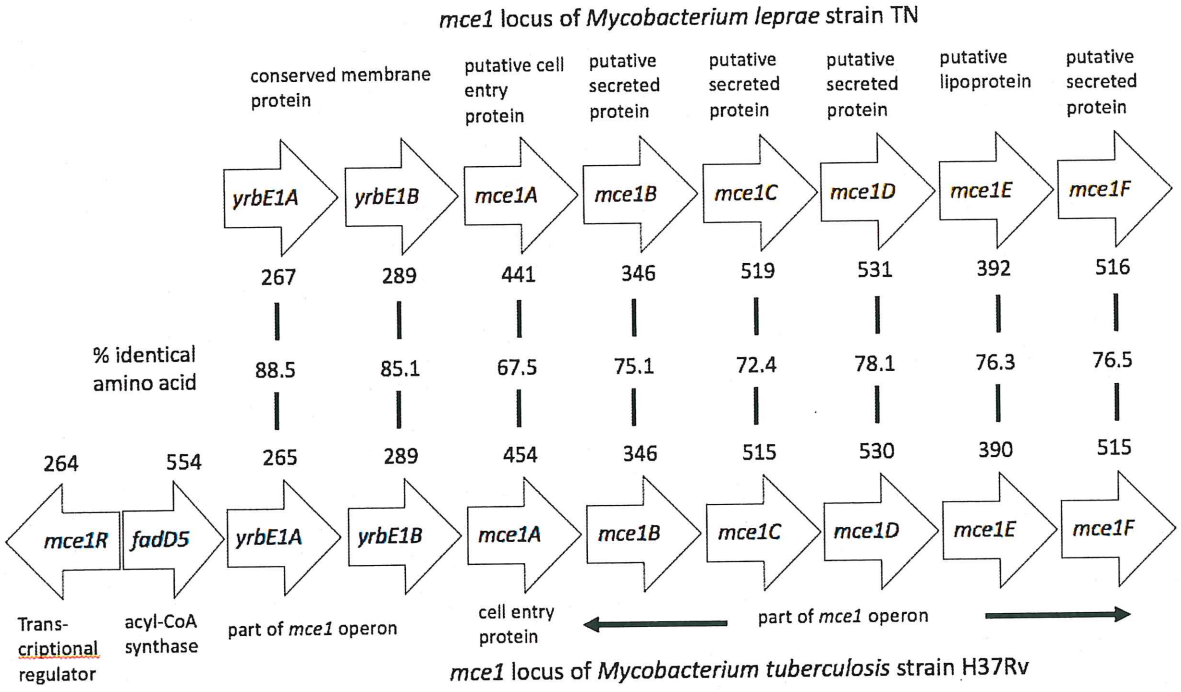

Fig 8. Comparison of the *mce1* locus of *Mycobacterium leprae* strain TN and *Mycobacterium tuberculosis* strain H37Rv.

The genome of *M. tuberculosis* strain H37Rv contains four *mce* operons (*mce1, 2, 3* and *4*), however, only one *mce* operon is conserved in the genome of *M. leprae* strain TN. The *mce1* operon is comprised of 8 gene (*yrbE1A, yrbE1B, mce1A, mce1B, mce1C, mce1D, mce1E* and *mce1F*). *M. leprae mce1A* gene is highly homologous (67.5%) to *M. tuberculosis mce1A* gene associated with mammalian epithelial cell entry and intracellular survival inside macrophages. Upper and bottom columns indicate the genes in *mce1* locus of *M. leprae* and *M. tuberculosis*, respectively. The numbers representing in upper and bottom columns indicate amino acid residues corresponding to each gene. The numbers representing in middle column indicate percentages of amino acid identical to each set of genes. All of genetic information indicated in this figure were obtained from the web sites of Institut Pasteur (http://genolist.pasteur.fr/) and Sanger Centre (http://www.sanger.ac.uk/).

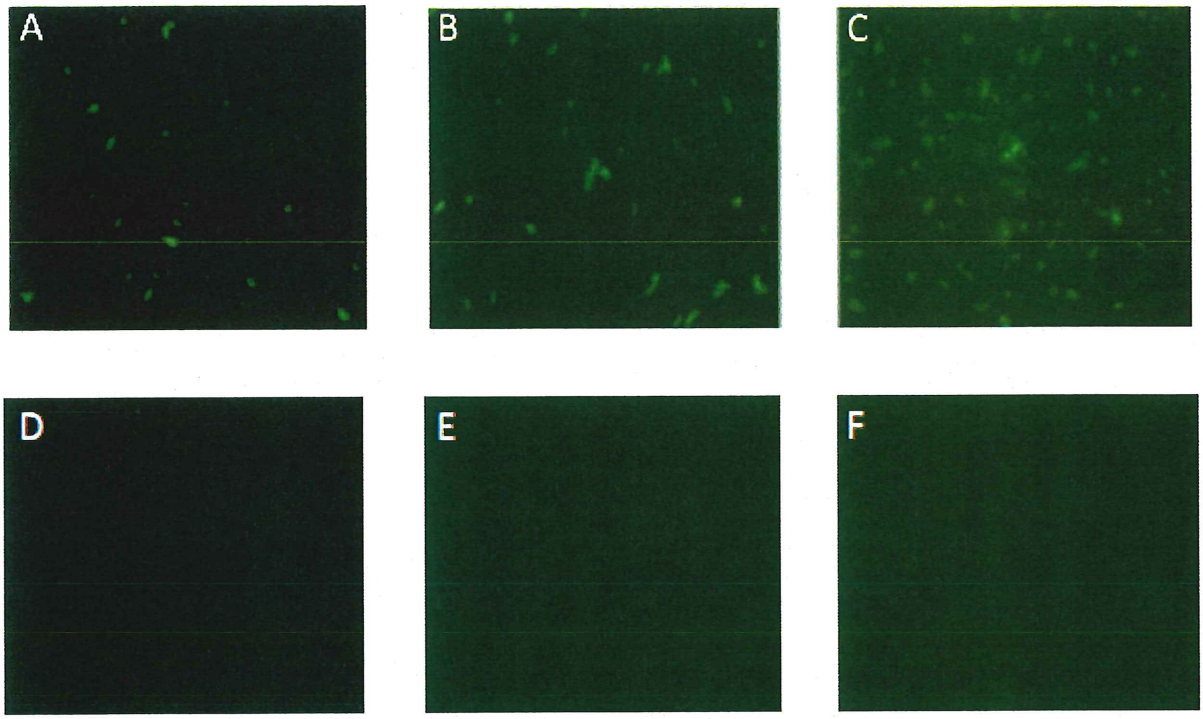

Fig 9. Indirect immunofluorescence staining of InvX, InvY and InvZ expressing *E. coli*.

invX, *invY* and *invZ* is presented on the surface of *E. coli* cells by the AIDA autotransporter translocator. *E. coli* cells were surface labeled with a mouse polyclonal Ab raised against MceIA and a FITC-conjugated anti-mouse secondary Ab. Fluorescence microscopy showed that *E. coli* expressing the InvX, InvY and InvZ -AIDA fusion protein bound anti-MceIA Ab. (A) InvX, (B) InvY, (C) InvZ. (D), (E) and (F) are controls of InvX, InvY and InvZ, respectively where normal mouse serum is used.

InvXa

M. tuberculosis : N terminus – VNADIKATTVFGGKYVSLTTPKNP – C terminus
M. leprae : N terminus – VNADIKATTVFGGKYVSLTTPEHP – C terminus
% identical of InvXa = 91.6%

InvXb

M. tuberculosis : N terminus – TKRRITPKDVIDVRSVTTEINT – C terminus
M. leprae : N terminus – SQKRLTPQTVIDARSVTTEINT – C terminus
% identical of InvXb = 68.1%

InvXc

M. tuberculosis : N terminus – LFQTLTSIAEK – C terminus
M. leprae : N terminus – LFQTITLIAAK – C terminus
% identical of InvXc = 72.7%

InvXd

M. tuberculosis : N terminus – VDPVKLNLTLSAAAE – C terminus
M. leprae : N terminus – VDPIKLNLTLSAAAQ – C terminus
% identical of InvXd = 86.6%

Fig 10. Comparison of amino acid sequences in InvX between *M. leprae* and *M. tuberculosis* Mce1A.