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1 **The unique tropism of *Mycobacterium leprae* to the nasal epithelial cells can be explained**
2 **by the mammalian cell entry protein 1A**
3
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21 **Short title: Nasal epithelial cell invasion of *Mycobaterium leprae***
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25 # Abstract

26 Leprosy is a chronic infection where the skin and peripheral nervous system is invaded
27 by *Mycobacterium leprae.* The infection mechanism remains unknown in part because
28 culture methods have not been established yet for *M.leprae.*

29 Mce1A protein (442 aa) is coded by mce1A (1326 bp) of *M.leprae*. The mce1A homolog
30 in *Mycobacterium tuberculosis* is known to be associated with *M.tuberculosis* epithelial cell
31 entry, and survival and multiplication within macrophages. Studies using recombinant
32 proteins have indicated that mce1A of *M.leprae* is also associated with epithelial cell entry.
33 This study is aimed at identifying particular sequences within mce1A associated with
34 *M.leprae* epithelial cell entry.

35 Recombinant proteins having N-terminus and C-terminus truncations of the mce1A
36 region of *M.leprae* were created in *Eschericia coli.* Entry activity of latex beads, coated with
37 these truncated proteins (r-lep37kDa and r-lep27kDa), into HeLa cells was observed by
38 electron microscopy. The entry activity was preserved even when 315 bp (105 aa) and 922
39 bp (308 aa) was truncated from the N-terminus and C-terminus, respectively. This 316 – 921
40 bp region was divided into three sub-regions: 316 – 531 bp (InvX), 532 – 753 bp (InvY), and
41 754 – 921 bp (InvZ). Each sub-region was cloned into an AIDA vector and expressed on the
42 surface of *E.coli.* Entry of these *E.coli* into monolayer-cultured HeLa and RPMI2650 cells
43 was observed by electron microscopy. Only *E.coli* harboring the InvX sub-region exhibited
44 cell entry. InvX was further divided into 4 domains, InvXa - InvXd, containing sequences 1
45 – 24 aa, 25 – 46 aa, 47 – 57 aa, and 58 – 72 aa, respectively.

46 Recombinant *E.coli*, expressing each of InvXa - InvXd on the surface, were treated with
47 antibodies against these domains, then added to monolayer cultured RPMI cells. The
48 effectiveness of these antibodies in preventing cell entry was studied by colony counting.
49 Entry activity was suppressed by antibodies against InvXa, InvXb, and InvXd. This suggests

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that these three InvX domains of mce1A are important for *M.leprae* invasion into nasal epithelial cells.

Author Summary

Mce1A protein is encoded by the mce1A region of mce1 locus of *M.tuberculosis* and *M.leprae*, and is involved in the bacteria's invasion into epithelial cells. The present study revealed that the active sequence of *M.leprae* involved in the invasion into nasal mucosa epithelial cells is present in the 316-531 bp region of mce1A.

The most important region of mce1A protein involved in the invasion of *M.tuberculosis* into human epithelial cells is called the InvIII region, which is located between amino acids at position 130 to 152. The InvIII region of *M.tuberculosis* corresponds to InvXb of *M.leprae*. The sequences of these regions are identical between amino acids at positions 10 to position 22 as counted from the N terminus, except that amino acids at positions 1 to 3, 5, 8, 9, 13 are different between *M.leprae* and *M.tuberculosis*. 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 *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 indicating that in *M.leprae*, far more proteins are inactivated as compared to *M.tuberculosis*. The present study also revealed that, as in *M.tuberculosis,* the mce1A protein is expressed on the surface of bacteria as a native protein. In light of these data, the mce1A protein is considered to be one of the most important proteins involved in the invasion of *M.leprae* into nasal mucosa epithelial cells.

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

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74 number of Hansen's disease cases has drastically decreased in developed countries, worldwide,
75 the number of new cases of Hansen's disease has only dipped below 200,000 per year.
76 Hansen's disease is one of the Neglected Tropical Disease (NTDs) and is still a major problem
77 against public health[1].

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

91 *M.leprae* cannot be artificially cultured. One possible reason for this is the presence of a
92 large number of pseudogenes. *M.leprae* has various enzyme-coding genes that are replaced
93 with pseudogenes, and therefore has only a minimum metabolic activity and multiplies in
94 macrophages and Schwann cells. Invasion mechanism of *M.leprae* into Schwan cells have been
95 studied by Rambukkana, et al., in details. The study revealed that laminin-2 present in the basal
96 lamina surrounding Schwann cells serves as a receptor, and histone-like protein Hlp/LBP
97 expressed on the surface of the bacteria binds to phenolic glycolipid PGL-1, making the entry
98 into the cell possible[11-14].

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99 To infect Schwann cells, *M.leprae* has to invade the epidermal cells first. The mechanism
100 of *M.leprae* invasion into the epidermal cells, however, has not been elucidated yet. Meanwhile,
101 gene regions involved in the invasion of *Mycobacterium tuberculosis (M.tuberculosis)* into
102 epidermal cells are already known[15,16]. Casali et al. reported that, using adhesin involved in
103 diffuse adherence (AIDA) method, the region coded for by 316 - 531 bp of *M.tuberculosis*
104 mce1A region (Rv 0169; 198534 - 199898 bp, 1365 bp) is expressed on the surface of *E.coli*
105 as a polypeptide chain, thereby imparting the *E.coli* with the ability to invade HeLa cells, that
106 the invasion activity is inhibited by the monoclonal antibody that recognizes the continuous
107 peptide of InvIII region (388 – 453 bp)[17,18]. It became clear that *M.leprae* includes a region
108 (ML2589, 1326 bp) highly homologous to mce1A protein of *M.tuberculosis.* Sato et al.
109 reported that a recombinant protein, a 37 kDa protein encoded by 73 - 921 bp, which is the
110 mce1A region excluding the signal sequence, was found to have an invasion activity into
111 epidermal cells[19]. However, the active sequence involved in the invasion by *M.leprae* into
112 epidermal cells has not been identified. The present study was conducted to identify the active
113 sequence in the mce1A region. In this study, the N-terminus and C-terminus truncated proteins
114 expressed on the *E.coli,* where *E.coli* with specific regions are expressed thereon by the AIDA
115 method, and hyperimmune antisera against the invasion region are used to investigate the
116 invasion activity into epidermal cells.

117 Material and methods

118 Bacterial strains and plasmid

119 The genomic DNA used in the study was isolated from *M.leprae* strain Thai 53, which
120 was maintained at Leprosy Research Centre, National Institute of Infectious Diseases, Japan,
121 as previously described[20,21]. The pQE30 plasmid and *E.coli* M15 (pREP4) were purchased

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122 from Qiagen (Valencia, CA). The pQE30 plasmid was used as expression vector. *E.coli* M15
123 (pREP4) was used as a host for the vector, as recommended by the manufacturer.
124 The pMK90 plasmid and *E.coli* UT4400 were obtained from Dr. Riley (University of
125 California at Berkeley, California, USA).

126 ## Construction of vector

127 In Sanger Center *M. leprae* strain TN complete genome sequence, mce1A gene is a 1326
128 bp putative ORF located between positions 3092446 and 3093771 (NCBI-GeneID: 910890).
129 The mce1A DNA sequence of strain Thai 53 was identical to that of strain TN. It was subcloned
130 into pQE30 vector in a truncated reading frame. The 603 bp ORF deleted at 5' and 3' ends of
131 mce1A gene is located between positions 316 and 921 (Fig 1). This sequence was amplified
132 by polymerase chain reaction (PCR) directly from the genomic DNA of *M. leprae* strain Thai
133 53 with oligonucleotide primers designed to introduce SacI and HindIII endonuclease
134 restriction sites at the ends. The amplified products were ligated into the pQE30 vector
135 linearized with SacI and HindIII. The use of pQE30 vector allowed the plasmid to express the
136 mce1A product with a polyhistidine (6 x His) tag at the N-terminus (r-mce1Ap). The resultant
137 plasmid was cloned into *E.coli* M15 (pREP4) by electroporation (Gene Pulser II, Bio-Rad,
138 Hercules, CA), according to the manufacturer's instructions.
139

140 **Fig 1. r-mce1A truncated protein regions expressed on *E.coli*, regions externally**
141 **expressed using AIDA method, and regions for which hyperimmune antisera is prepared.**
142 Using r-lep45kDa protein encoded in the entire region of mce1A minus signal sequences (73
143 – 1326 bp) as a reference, the protein with its C terminus truncated to 921 bp was labeled as r-
144 lep37kDa protein, and the protein with its N terminus truncated to 315 bp was labeled as r-
145 lep27kDa protein. Furthermore, 316 – 921 bp region was divided into 316 – 531 bp, 532 – 753
146 bp, and 754 – 921 bp, and recombinant *E.coli* in which these regions are externally expressed

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147 using AIDA method were prepared. InvX region (316 – 531 bp) was further divided into InvXa
148 (316 – 387 bp), InvXb (388 – 453 bp), InvXc (454 – 486 bp), InvXd (487 – 531 bp), and
149 hyperimmune antisera for each region was prepared.

150

151 Plasmid pMK90 is an ampicillin-resistant pBR322 derivative that expresses a
152 recombinant AIDA protein under the control of its own promotor[22]. The AIDA coding
153 sequence has been altered to remove the native passenger; it consists of a 49-amino-acid signal
154 peptide. A 78-amino-acid linker region incorporating a multiple cloning site, and the entire
155 440-amino-acid barrel core. A 216 bp DNA fragment encoding InvX (*M.leprae* positions
156 3092761 – 3092976 bp), 222 bp DNA fragment encoding InvY (*M.leprae* positions 3092977
157 – 3093198 bp), 168 bp DNA fragment encoding InvZ (*M.leprae* positions 3093199 – 3093366
158 bp) was amplified by PCR from a plasmid containing mce1A and cloned into pMK90,
159 generating pMK100. The correct insert was confirmed by sequencing. The amino acid
160 sequence of InvX is
161 VNADIKATTVGGKYVSLTTPPEHPSQKRLTPQTVIDARSVTTEINTLFQTITLIAEKVD
162 PIKLNLTLSAAAQ (316 – 531 bp), the amino acid sequence of InvY is
163 SLAGLGERFGQSIVNGNSVLDDVNSQLPQARHDIQQLASLGDTYANSASDFFDFLNN
164 SIVTSRTI (532 – 753 bp), and the amino acid sequence of InvZ is
165 VLLAAVGFGNTGADIFSRSGPYLARGAADLVPTAQLLDTYSPAIFCTLRNYHDIEP
166 (754 – 921 bp) (Fig 1).

167 Protein expression and purification

168 Recombinant protein was expressed and purified according to manufacture's instruction.
169 Briefly, *E.coli* M15 [pREP4] containing pQE30/mce1A plasmid was grown overnight in 10-
170 ml superbroth containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. A 500 μl aliquot of
171 bacterial suspension was pelleted, resuspended in 30 ml of superbroth and incubated at 37°C

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172 for 1 – 2 h until OD_{600} = 0.6. Then isopropyl β-D-thiogalactoside was added to final
173 concentration 1 mM and incubated for 3 h at 37°C. The induced and uninduced r-*E.coli* strains
174 were analyzed by SDS-polyacryamide gel electrophoresis (SDS-PAGE). The newly expressed
175 protein formed an inclusion body in the r-*E.coli* host. The inclusion body was therefore purified
176 under denaturing conditions according to the instructions of the expression vector's respective
177 manufactures. The 6 x His tag mce1A solubilized with lysis buffer (6 M Guanidine, 10 mM
178 Tris—HCl, 100 mM NaH_2PO_4, pH 8.0) was bound to a Ni—NTA resin column equilibrated
179 with lysis buffer, and was eluted by elusion buffer (6 M Guanidine, 10 mM Tris—HCl, 100
180 mM NaH_2PO_4, 20—250 mM imidazole, pH 6.3). The eluted protein were subsequently
181 refolded with 1 mM dithiothreitol (Sigma, St. Louis, MO, USA) and 0.1 mM
182 phenylmethylsulfonyl fluoride (Sigma) by dialysis, gradually removing guanidine. The r-
183 mce1A was finally purified and refolded as a soluble protein. The protein was separated by
184 SDS-PAGE and was analyzed for purity by Coomasie brilliant blue R-250 staining.

185 **Immunoelectron microscopy**

186 The antibody against the mce1A protein was prepared in Balb/c mice (a 45 kDa
187 recombinant mce1A protein prepared previously using *E.coli* was used as the immunogen).
188 The r-45kDa-mce1A protein was used for antibody production because it was most abundantly
189 expressed in the *E.coli* host that we used. The r-45kDa-mce1A protein was mixed with Titer
190 Max Gold (AdipoGen Life Sciences, Liestal, Switzerland) of the same amount. Approximately
191 100 μg of the protein was administered subcutaneously at five sites in four 7-weeks-old Balb/c
192 mice, followed by two booster injections of 100 μg each 2 and 4 weeks after the first injection.
193 Blood was harvested 2 weeks after the last booster dose.

194 The antiserum was used in the colloidal gold immunoelectron microscopy. A bacterial
195 pellet (containing ≈ 10^7 organisms) of *M.leprae* strain Thai 53 that that had been multiplied in
196 footpads of athymic nude mice, was fixed in 3% glutaraldehyde in phosphate buffer saline

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197 (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
198 dilution of the mice antibody raised against mce1A. The suspension was then washed and
199 incubated at 4°C for 16 h with colloidal gold suspension containing 5 nm gold particles (1.9 ×
200 10^{13} particles ml^{-1}) conjugated to anti-mice IgG goat antibody (Amersham/GE Health Care
201 Life Science, Tokyo, Japan). The cells were washed again five times in PBS, stained with 0.1%
202 uranyl acetate in water and examined with a HITACHI model H-15 electron microscope.

203 Cell Culture

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

214 Cell uptake assay of protein-coated latex beads by electron 215 microscopy

216 A 30 µ of stock suspensions of 1.1 µm diameter polystyrene latex beads, containing 5 ×
217 10^8 beads/ml (Sigma), were mixed in 150 µl of PBS containing 50 µg/ml of each set of protein
218 and incubated for 16 h at 37°C. After incubation, the samples were centrifuged at 7000 × g and
219 resuspended in 750 µl of PBS. A 500-µl sample of this suspension was added to a near-
220 confluent cultured cell monolayer grown in a 25-cm^2 flask containing 7 ml of appropriate media

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221 for cultured cells. The cells were incubated for 5 h at 37°C in a CO_2 incubator, washed four
222 times with PBS and one time with 0.1 M cacodylate phosphate buffer (pH 7.6), and then
223 collected with cell-scraper (Becton Dickinson, Japan). The collected cells were fixed with 2%
224 glutaraldehyde in 0.1 M cacodylate phosphate buffer (pH 7.6) at 4°C overnight, post-fixed with
225 1% osmium tetroxide in PBS, dehydrated through graded ethanol solutions and embedded in
226 Spurr's low-viscosity embedding media. The ultrathin sections were stained with uranyl acetate
227 and lead citrate and examined with a JEM-1200EX (JEOL, Tokyo, Japan) transmission
228 electron microscope. Coated beads with bovine serum albumin (BSA) fraction V (Boehringer
229 Mannheim, GmbH, Germany) were used as negative controls.

230 Immunofluorescence microscopy

231 *E.coli* cells were fixed onto microscope slides with 0.4% paraformaldehyde for 10 min at
232 room temperature, and non-specific binding was blocked by incubation in 1% (wt/vol) BSA
233 for 30 min. Slides were incubated for 1 h with a 1:200 dilution of a rabbit antibody raised
234 against InvXa, InvXb, InvXc, and InvXd washed, and incubated with 1:1000 dilution of
235 fluorescein isothiocyanate-labeled anti-rabbit antibody (Abcam Plc, Cambridge, UK) for 30
236 min. After extensive washing, the coverslips were mounted. Slides were viewed on an Olympus
237 BX51 inverted microscope with an epifluorescence attachment.

238 Invasion assay

239 InvX, InvY, InvZ mediated invasion of *E.coli* into the nasal epithelial cells
240 by electron microscopy

241 The culture medium of a monolayer culture of 1.15×10^6 HeLa cells and 3×10^7 RPMI2650
242 cells, was replaced with a culture medium that does not contain antibiotic substances. Then
243 *E.coli* externally expressing by AIDA (UT4400/lep316, UT4400/lep532, UT4400/lep754) was
244 added at a bacteria to cells ratio of 100:1, and incubated in a CO_2 incubator at 37°C, for 9 h for

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245 HeLa cells and 6 h for RPMI2650 cells. After culturing, the cell surface was washed with PBS,
246 and then harvested using a cell scraper.
247 Infected cells were prepared for examination by transmission electron microscopy as
248 previously described[16]. Briefly, cells were fixed in 2% glutaraldehyde and stained with
249 osmium tetroxide solution before dehydration through graded ethanol solutions. Cells were
250 embedded in Spurr's low-viscosity embedding medium, ultrathin sections were stained with
251 uranyl acetate and lead citrate. Samples were examined with a JEM-1230 (JEOL) transmission
252 electron microscope.

253 **Gentamicin protection assays**

254 Gentamicin protection assays were performed according to the method of Elsinghorst[23].
255 RPMI2650 cells were seeded at 5×10^5 cells per well directly into 24-well plates and cultured
256 for 24 h until confluent. Cell culture medium was modified to contain no antibiotics.
257 Recombinant *E.coli* cells were added to the monolayer at a multiplicity of infection (MOI) of
258 10:1 and incubated at 37°C for 3 h. To enumerate intracellular bacteria, the monolayer was
259 washed five times with PBS and incubated with medium containing 100μg of GM (Sigma) per
260 ml for 2 h to kill extracellular bacteria and permit the enumeration of intracellular bacteria. The
261 monolayer was again washed five times with PBS and lysed with 0.1% Triton X-100 (Eastman
262 Kodak, Rochester, NY). Serial dilutions of released bacteria were plated for counting. Results
263 shown are the mean values for an experiment performed in triplicate. Each experiment was
264 performed three times using independent cultures, with similar result.

265 **Inhibition Assay**

266 InvXa, InvXb, InvXc, and InvXd antibodies were added in the amount of 1/200 (200 μg)
267 to *E.coli* that externally express UT4400/lep316 by AIDA adjusted to 1×10^8 CFU/ml. This
268 was allowed to react on a rotating platform at 4°C overnight to make antibody-treated bacteria.

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269 *E.coli* externally expressing proteins by AIDA were allowed to react with IgG from healthy,
270 control rabbits in a similar manner as the control, where this was used as the bacteria untreated
271 by antibody. After the medium for RPMI2650 cells, which were monolayer-cultured in a 24-
272 well plates, 5×10^5 cells/well, was replaced with a medium not containing antibiotic agent, the
273 antibody-treated bacteria and untreated bacteria were added at a bacteria to cells ratio of 30:1.
274 After culturing in CO_2 incubator at 37°C for 3 h, the surface of the cells were washed with PBS
275 five times, and the medium was replaced with a 100 μg/ml GM-appended DME medium to kill
276 the bacteria outside the cells, followed by additional incubation for 2 h. The surface of cells
277 was washed with PBS, and then 0.1% Triton X-100-added PBS was added in the amount of 1
278 ml/well to break the cells and the bacteria inside the cells were harvested. The harvested
279 bacteria suspension liquid was serially diluted 10 times with PBS, and then was applied to
280 Heart Infusion agar medium (Nissui, Tokyo, Japan). This was left overnight at 37°C, and then
281 the colonies were counted to determine the number of bacteria entered into the cells. The
282 cultured cells were prepared in the amount of 3 wells each, and the average of each well and
283 standard deviation were calculated and the result was presented on a graph.

284 Ethics statement

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

288 Result

289 Immunoelectron microscopy of *M.leprae*

290 Immunoelectron microscopy was employed to determine whether recombinant *E.coli*
291 expressed the mce1A protein on the cell surface.

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292 The bacilli expressing mce1A protein were pretreated with an antibody (Ab) raised against
293 r-45 kDa mce1A protein, and were followed by incubation with anti-IgG Ab-conjugated
294 colloidal gold particles (Fig 2). The immunoelectron microscopic study revealed that the native
295 mce1A protein is expressed on the surface of bacilli. This confirms that the recombinant *E.coli*
296 not only expresses mce1, but the mce1 is transported to the cell surface and sufficiently
297 presented such that it can bind the antibody against it.
298
299 **Fig 2. Colloidal gold immunoelectron microscopic analysis of *M.leprae* strain Thai 53.** The
300 bacilli were pretreated with an antibody (Ab) raised against r-45 kDa mce1A protein, and were
301 followed by incubation with anti-IgG Ab-conjugated colloidal gold particles. Gold particles
302 are shown decorating the surface of the *M.leprae* bacillus (arrowheads).
303

304 HeLa cell uptake assay of protein-coated polystyrene latex 305 microbeads by electron microscopy

306 The active sequence involved in the invasion into the epithelial cells was investigated in
307 the following manner. The r-lep37kDa protein, which had been prepared in the previous
308 experiment using r-lep45kDa protein as the reference by truncating the C terminus to 308 aa
309 (922 bp), was further truncated to 105 aa (315 bp) from N terminus to provide r-lep27kDa
310 protein where the proteins using were expressed using an *E.coli* expression system (Fig 1).
311 Each of the truncated protein was observed for invasion activity into HeLa cells using an
312 electron microscope. In this observation, images of beads coated with r-lep37kDa protein and
313 beads coated with r-lep27kDa protein invading into the cytoplasm of HeLa cells were captured,
314 but BSA-coated beads, which are the negative control, were not found to invade the cytoplasm
315 (Fig 3). This result suggest that the active sequence is present between 316 – 921 bp, which
316 encodes r-lep27kDa protein.

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317

318 **Fig 3. HeLa cells uptake assay of protein-coated polystyrene latex microbeads by electron**
319 **microscopy.** Monolayer-cultured HeLa cells and the truncated protein-coated beads and BSA-
320 coated beads were allowed to react for 5 h, and the entry of beads into HeLa cells were observed
321 under an electron microscope. As shown in the arrow, (A) r-lep37kDa protein-coated beads
322 and (B) r-lep27kDa protein-coated beads were observed to enter into HeLa cells, but (C) BSA-
323 coated beads were not observed to enter into HeLa cells. Magnification ×3500 (A), ×5000
324 (B,C)

325

326 InvX, InvY, InvZ mediated invasion of *E.coli* into the nasal 327 epithelial cells by electron microscopy

328 The active sequence was further inverstigated. The 316 – 921 bp region was divided into
329 InvX; 316 – 531 bp, InvY: 532 – 753 bp, InvZ: 754 – 921 bp, and each of the regions was
330 incorporated into AIDA vector to produce a recombinant *E.coli* externally expressing the
331 proteins (Fig 1). The *E.coli* externally expressing the proteins by the AIDA method were
332 observed for invasion activity into HeLa cells and RPMI2650 cells under the electron
333 microscope. *E.coli* expressing InvX (UT4400/lep316) was found in abundance in the
334 cytoplasm. *E.coli* expressing InvY and InvZ (UT4400/lep532 and UT4400/lep754) and
335 UT4400 were observed present around the cells but not inside the cytoplasm (Fig 4). These
336 results suggest that the active sequence is present in 316 – 531 bp (InvX).

337

338 **Fig 4. Transmission electron microscopy of InvX, InvY, InvZ mediated invasion of *E.coli***
339 **into HeLa and nasal epithelial cells.** UT4400 (A,E), UT4400/lep316 (B,F), UT4400/lep532
340 (C,G), and UT4400/lep754 (D,H) were added to monolayer cultured HeLa cells (1) and to
341 RPMI2650 cells (2) at a cell to bacteria ratio of 1:100. They were allowed to react for 9 h and

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342 6 h, respectively, and *E.coli* entry into the cells was observed under an electron microscope.
343 Only UT4400/lep316 (B,F) was observed to invade HeLa cells and RPMI2650 cells. Although
344 UT4400 (A,E), UT4400/lep532 (C,G) and UT4400/lep754 (D,H) were observed to be present
345 around the cells, no invasion into the cells was observed with them. Magnification ×5000

346 InvX, InvY, InvZ mediated invasion of *E.coli* into the nasal 347 epithelial cells (gentamicin protection assay)

348 Next, using a gentamicin protection assay, the number of bacteria which entered into
349 RPMI2650 cells was determined in colony forming units (CFU).
350 To determine uptake of the host *E.coli* cells using a gentamicin protection assay, we
351 assessed the invasive ability of InvX, InvY, InvZ expressing *E.coli* (pMK100) cells showed
352 invasion levels at the 3 h time point.
353 In RPMI2650 cells, invasive activity of InvX-expressing *E.coli* was significantly higher
354 than that of InvY, InvZ, and negative control (Fig 5). The result was similar to the observations
355 by electron microcopy. Invasion activity into nasal mucosa epithelial cells was successfully
356 imparted to a pathogenic *E.coli* by externally expressing the InvX region of *M.leprae* on the
357 *E.coli*. The InvX mediates the nasal epithelial cells invasion by non-pathogenic *E.coli*. The
358 InvX region within mce1A protein is then sufficient for the invasion of *E.coli* into the cells.
359

360 **Fig 5. InvX, InvY, InvZ mediated invasion by *E.coli* into the nasal epithelial cells**
361 **(gentamicin protection assay)**. Each of AIDA externally expressing *E.coli* was added to
362 manolayer cultured RPMI2650 cells at a cells to bacteria ration of 1:10 and the mixture was
363 allowed to react for 3 h. The bacteria outside the cell were subjected to disinfection by GM for
364 2 h. The bacteria inside the cell were counted with the colony count method, which was
365 presented as CFU. Bars represent the mean ± S.D. of intracellular bacteria as a CFU in a

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366 representative experiment performed in triplicate. Asterisks indicate significant difference
367 compared with an IgG control (Scheffe's multiple comparison test).
368

369 Indirect immunofluorescence staining of InvX expressing *E.coli* by 370 antibodies corresponding to each region of mce1A

371 Indirect immunofluorescence was used to determine which regions of mce1A are sufficient
372 to confer invasive ability to *E.coli*.
373 A 72-amino acid fragment of the InvX region was divided into four regions, InvXa (24 aa),
374 InvXb (22 aa), InvXc (11 aa), and InvXd (15 aa). These peptides were subsequently synthetized
375 as immune antigens for anti-InvX antibodies (anti-InvXa Ab, anti-InvXb Ab, anti-InvXc Ab,
376 and anti-InvXd Ab) were studied.
377 In order to examine whether the antibodies recognize each of the regions, fluorescence
378 immunostaining was conducted on the antibodies. The result was the following. Fluorescence
379 microscopy revealed bacterial surface binding of the InvX antibodies by their binding of
380 labelled secondary antibodies of fluorescence goat anti-rabbit IgG InvXa , InvXb, InvXc, and
381 InvXd (Fig 6).
382

383 **Fig 6. Indirect immunofluorescence staining of InvX expressing *E.coli* by antibodies**
384 **corresponding to each region of InvX.** Visualization of the bacilli reveals bacterial surface
385 binding of the InvX antibody bound to fluorescent goat anti-rabbit IgG. (A) InvXa, (B) InvXb,
386 (C) InvXc, (D) InvXd.
387

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388 **Inhibitory effects of anti-InvX antibodies raised against each set of**
389 **synthetic peptide corresponding to an InvX devided region on the**
390 **nasal epithelial cells invasion of InvX expressing *E.coli***

391 In order to elucidate the role mce1A protein in association with the nasal epithelial cells
392 invasion of *M.leprae*, we analyzed inhibitory effects of the resultant antibodies on the cell
393 uptake of InvX-expressing *E.coli* by the inhibition assay. As shown in CFU analysis, the InvX-
394 expressing *E. coli* pretreated with anti-InvXa Ab, anti-InvXb Ab, and anti-InvXd Ab had
395 significantly lower entry than the IgG control, but there was no significant difference in
396 pretreatment with anti-InvXc Ab and IgG control (Fig 7). These findings suggest that the
397 invasion activity was most suppressed when using antibodies to cover the polypeptide chain
398 encoded by 316 – 387 bp and expressed on the surface of *E.coli*.
399

400 **Fig 7. Inhibitory effects on anti-InvX antibodies raised against each set of synthetic**
401 **peptide corresponding to an InvX divided region on the nasal epithelial cells invasion by**
402 **InvX epressing *E.coli.*** pMK100 *E.coli* strain were pretreated overnight with resultant
403 antibodies and normal IgG as a control. Each antibody preparation was added to monolayer
404 RPMI2650 cells and incubated for 3 h. After washing, GM was added to eliminate bacteria
405 outside the cells. Bars represent mean ± standard deviation CFU of pMK100 *E.coli* treated with
406 antibodies. The invasive activity was suppressed by antibodies against InvXa, InvXb, and
407 InvXd. Asterisks indicate $p<0.05$, NS: Not significant (Dunnet test). Bars represent the mean
408 ± SD of intracellular bacteria in CFU in a representative experiment performed in triplicate.
409 Asterisks indicate a significant difference compared with an IgG control.
410

411 Discussion

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412 A number of studies have been conducted on the infection mode of *M.leprae*. In 1955,
413 Khanolkar et al. reported that *M.leprae* infection of *M.leprae* occurs by normal skin contact[3].
414 However, in 1963 Weddell et al. revealed that the infection does not occur unless the bacteria
415 is inoculated under the skin[24]. Rees et al. induced immune suppressed mice to inhale an
416 aerosols containing *M.leprae* which successfully infected the mice via upper airway[4].
417 Following this, Chehl et al. revealed that transnasal infections of *M.leprae* of nude mice was
418 possible[5]. From these studies, it became clear that the infection from aerosol containing
419 *M.leprae* and through the nasal membrane can be established. However, as of today, only
420 limited studies have been conducted regarding molecular mechanisms involved in the invasion.
421 The mce region is present in tuberculosis complex such as *M.tuberculosis* and
422 *Mycobacterium bovis*, as well as in atypical mycobacteria such as *Mycobacterium avium* and
423 *Mycobacterium intracellulare*[25]. Chitale et al. revealed that this mce1A protein involved in
424 the invasion into epithelial cells is expressed only in tuberculosis complex[16]. We had found
425 that *M.leprae* has a region highly homologous with to the mce1A region of *M.tuberculosis*,
426 and so far have prepared a recombinant protein (mce1A protein) encoded by mce1A region
427 (ML2589; 3092446 to 3093771, 1326 bp) of *M.leprae* to investigate invasion activities to
428 epithelial cells[15,19]. In the present study, we have confirmed that the mce1A protein was
429 actually expressed, as a native protein, on the surface of *M.leprae*, and prepared a recombinant
430 protein by truncating the N terminus and C terminus of mce1A region of *M.leprae* to
431 investigate the invasion activity into the epithelial cells. As a result, it was found that invasion
432 activity is maintained even if 105 aa (315 bp) is truncated from N terminus and 308 aa (922
433 bp) is truncated from C terminus. Next, 316 bp to 921 bp region was divided into 3 parts, and
434 each part was incorporated into an AIDA vector, where each region was externally expressed
435 as a polypeptide chain to investigate whether the ability to invade can be imparted to non-
436 pathogenic *E.*coli. These *E.coli* which externally express the protein by AIDA method were

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437 examined for the invasion activity using RPMI2650 cells, where the results indicate that active
438 sequence of *M.leprae* involved in the invasion into nasal mucosa epithelial cells is present in
439 the 316 – 531 bp of mce1A region.

440 The most important region of mce1A protein involved in the invasion of *M.tuberculosis*
441 into human epithelial cells is called the InvIII cell and this is located between amino acids of
442 position 130 to position 152[26]. The InvIII region of *M.tuberculosis* corresponds to InvXb of
443 *M.leprae*. The sequence of the regions are identical between amino acid of position 10 to
444 position 22 counted from N terminus, except that amino acids at positions 1 to 3, 5, 8, 9, 13 are
445 different between *M.leprae* and *M.tuberculosis.* Suppression test results also indicated that the
446 most important region of mce1A protein of *M.leprae* involved in the invasion into human
447 epithelial cells is different from that of *M.tuberculosis.*

448 While *M.tuberculosis* has 3,959 protein-encoding genes and only 6 psedogenes, *M.leprae*
449 has only 1,604 protein-encoding genes but has 1,116 pseudogenes[27], indicating that in
450 *M.leprae*, far more proteins are inactivated as compared to *M.tuberculosis*. As in
451 *M.tuberculosis*, the mce1A protein is expressed on the surface of bacteria as a native protein,
452 and therefore the protein is considered as one of the most important proteins involved in the
453 invasion of *M.leprae* into nasal mucosa epithelial cells.

454 # Acknowledgement

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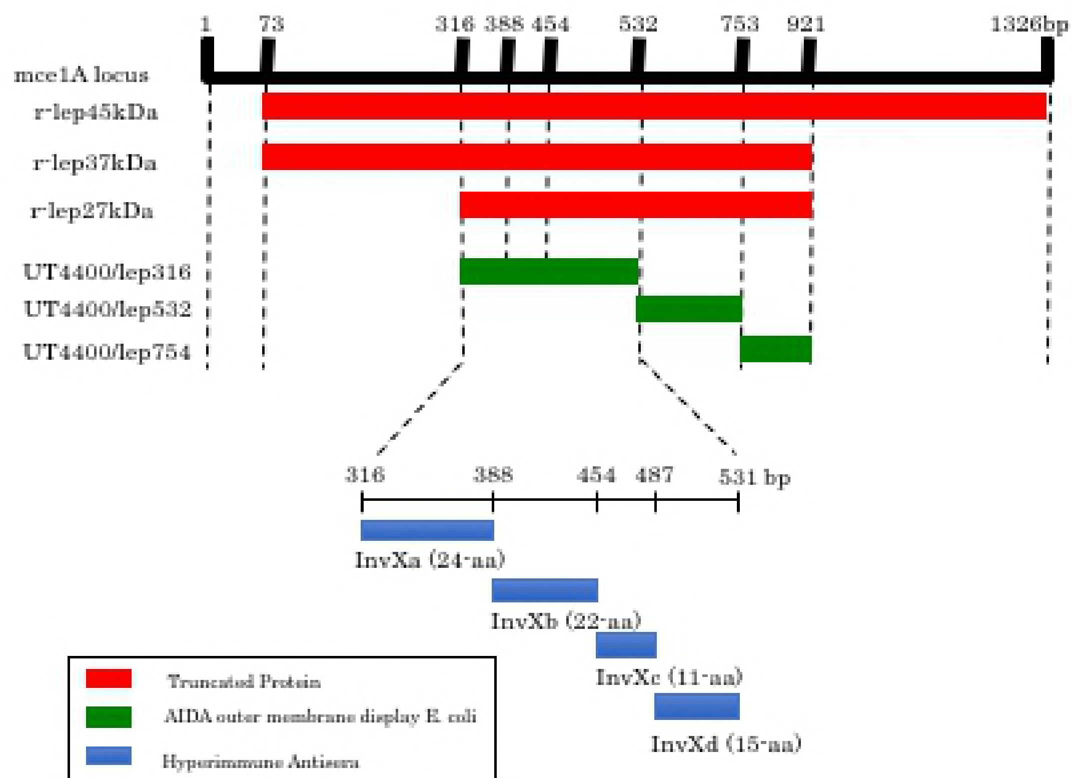
543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316
388
454
532
753
921
1326bp
mce1A locus
r-lep45kDa
r-lep37kDa
r-lep27kDa
UT4400/lep316
UT4400/lep532
UT4400/lep754
316
388
454
487
531 bp
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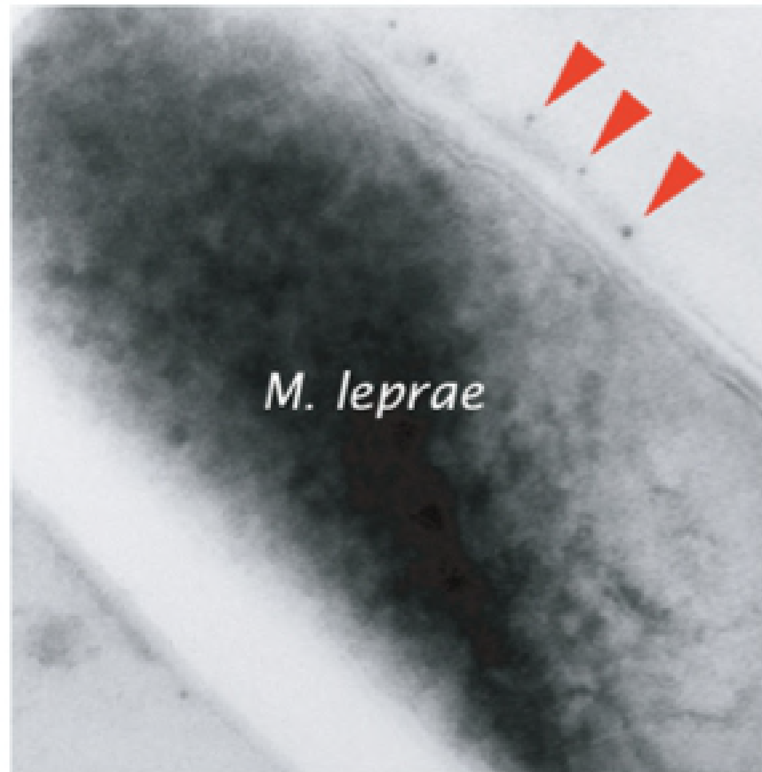
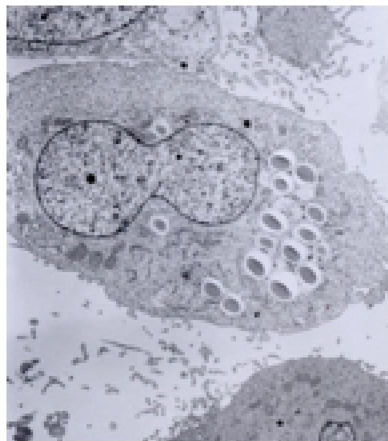
InvXb (22-aa)
InvXc (11-aa)
InvXd (15-aa)
Truncated Protein
AIDA outer membrane display E. coli
Hyperimmune Antisera


M. leprae

A



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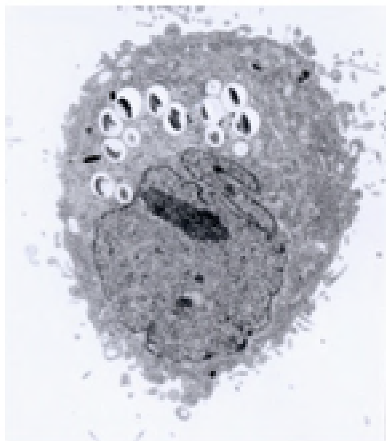

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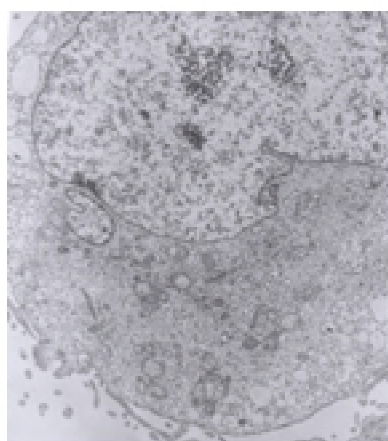

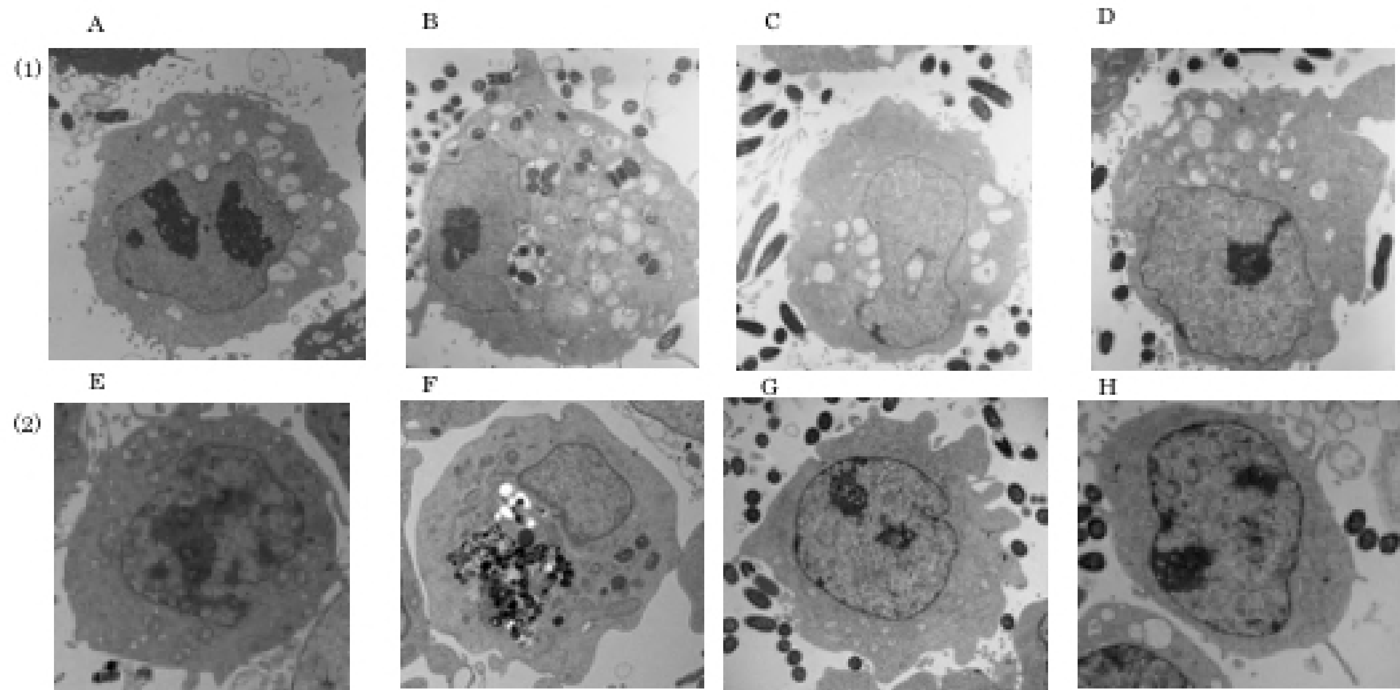
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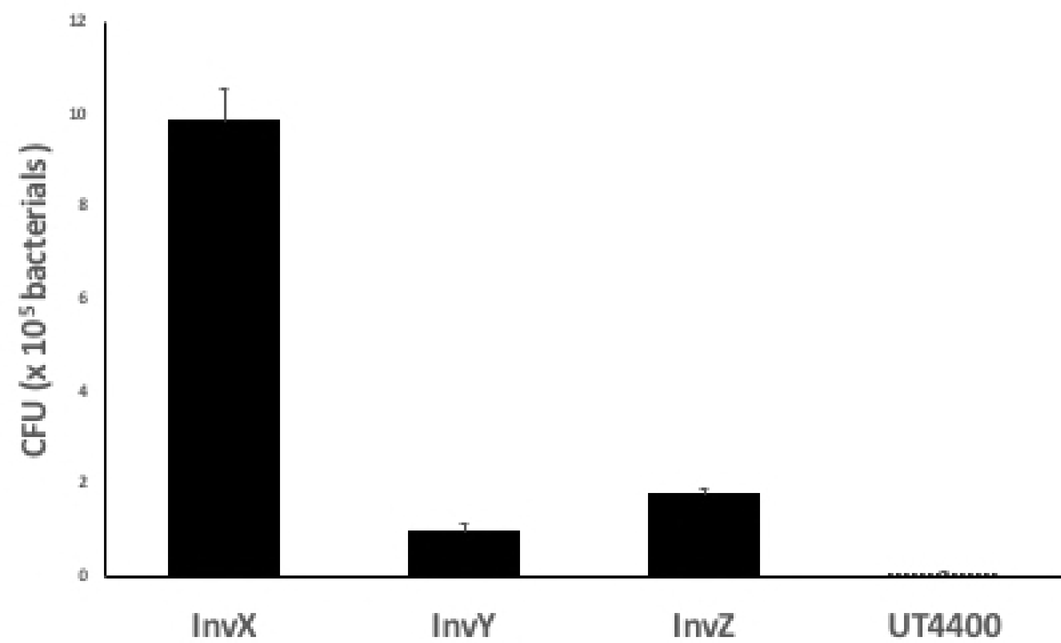
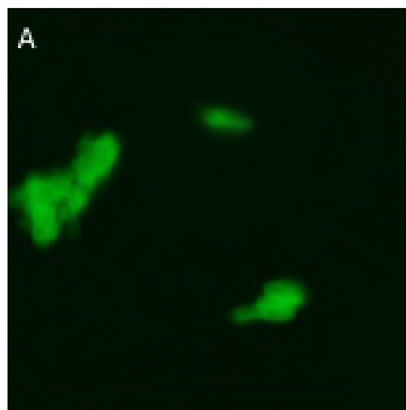


12
10
8
6
4
2
0
CFU (x 10⁵ bacterials)
InvX
InvY
InvZ
UT4400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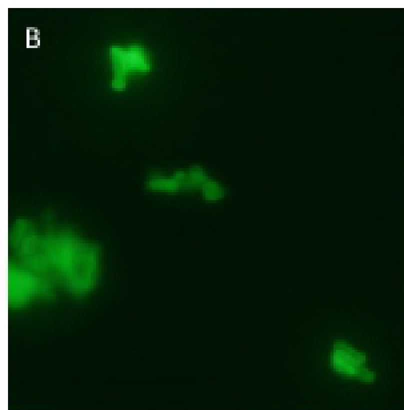
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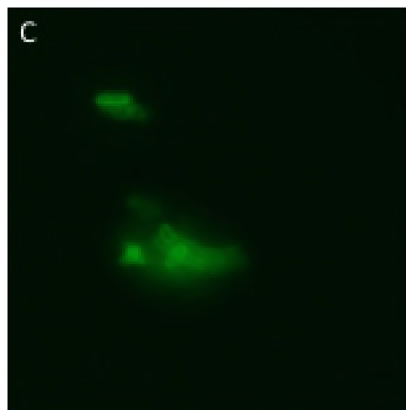
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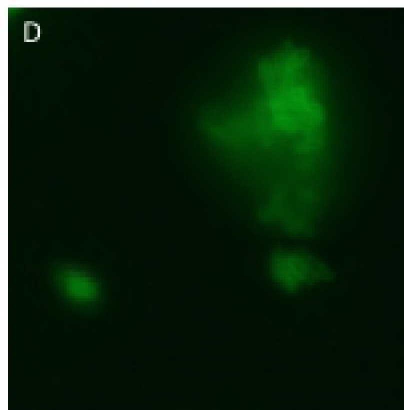
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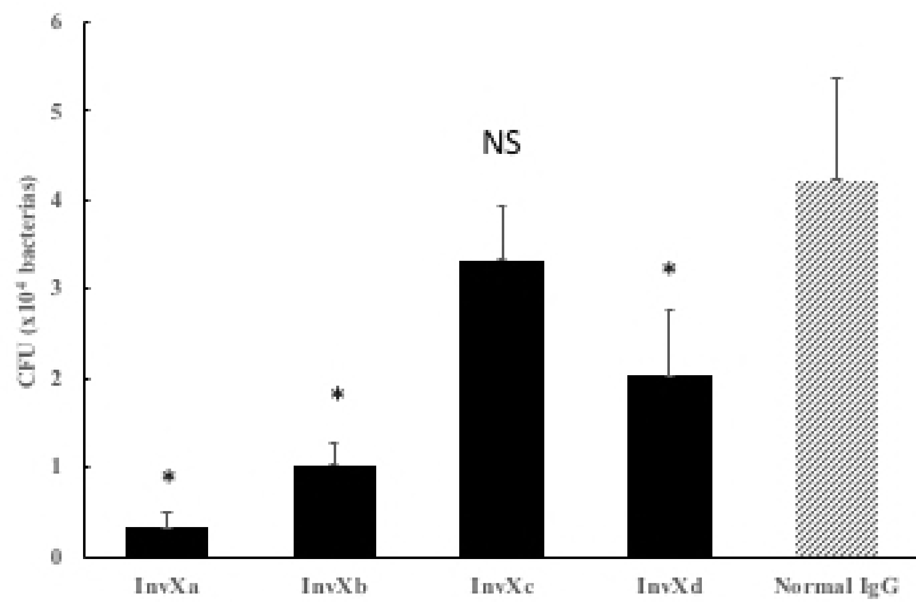
CFU (x10⁴ bacterias)
6
5
4
3
2
1
0
NS
*
*
*
InvXa
InvXb
InvXc
InvXd
Normal IgG