原　著

# 非貪食細胞に対する結核菌の侵入

[1]樋口　一恵　[1]原田　登之　[2]山田　博之　[3]小林　和夫
[4]竹田　稔

結核研究所基礎研究部 [1]免疫学科，[2]分子病理学科，
[3]大阪市立大学医学研究科感染防御学，[4]昭和大学医学部生化学教室

# THE INVASION OF *MYCOBACTERIUM TUBERCULOSIS* INTO NON-PHAGOCYTIC CELLS

[1]*Kazue HIGUCHI, [1]Nobuyuki HARADA, [2]Hiroyuki YAMADA,
[3]Kazuo KOBAYASHI, and [4]Minoru TAKEDA

*[1]*Divisions of Immunology and [2]Molecular Pathology, Department of Basic Research, Research Institute of Tuberculosis, [3]Department of Host Defense, Osaka City University Medical School, [4]Department of Biochemistry, Showa University School of Medicine*

To explore the ability of tubercle bacilli to invade and survive within non-phagocytic cells, we used in this study a human fibroblast cell line, WI-38, derived from normal embryonic lung and a human epithelial cell line, SQ-5, derived from lung squamous cell carcinoma. Live *M. tuberculosis* Erdman and *M. tuberculosis* H37Rv invaded WI-38 cells more efficiently than live *M. tuberculosis* H37Ra, *M. bovis* Ravenel, *M. bovis* BCG Tokyo and *M. bovis* BCG Pasteur. The capability of tubercle bacilli to invade WI-38 cells was Erdman ≧ H37Rv > BCG Pasteur ≒ *M. bovis* Ravenel ≒ BCG Tokyo > H37Ra. A similar invasive ability was observed using SQ-5 cells. In contrast with live bacilli, heat-killed bacilli failed to invade WI-38 cells, whereas they were detected within SQ-5 cells. These results and incorporation of latex beads suggest that SQ-5 cells, but not WI-38 cells, possess phagocytic activity. H37Rv multiplied most actively within WI-38 cells when compared to H37Ra and BCG Tokyo, suggesting that the ability to invade and survive within non-phagocytic cells reflects the more active invasion of virulent *M. tuberculosis* than avirulent *M.tuberculosis*. The assay system used in this study may help us to clarify the virulence of tubercle bacilli *in vitro*.

われわれは，ヒト正常胎児肺由来の繊維芽細胞株 WI-38 とヒト肺扁平上皮癌由来細胞株 SQ-5 を用い，非貪食細胞内へのヒト型結核菌，ウシ型結核菌および BCG の侵入能とその内部での生存能を調べた。ヒト型結核菌 Erdman および H37Rv の生菌は，ウシ型結核菌 Ravenel，ヒト型結核菌 H37Ra，BCG 東京株および BCG パスツール株の生菌より効率良く WI-38 に侵入した。

*〒204-8533 東京都清瀬市松山 3-1-24

*3-1-24, Matsuyama, Kiyose-shi, Tokyo 204-8533 Japan.
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生菌のWI-38への侵入能は，Erdman≧H37Rv＞BCGパスツール株≒*M. bovis* Ravenel≒BCG東京株＞H37Raの順であった。同様の侵入能は，SQ-5細胞を用いても得られた。生菌とは対照的に，熱処理死菌はWI-38細胞に取り込まれなかったが，SQ-5細胞には取り込まれた。これらの結果とラテックスビーズの取り込みの結果は，WI-38にはないがSQ-5細胞は貪食能を持っていることを示唆している。H37Rvは，H37RaおよびBCG東京株と比較しWI-38細胞内で最も多く増殖した。このことは，非貪食細胞内への侵入能とその内部での生存能は，弱毒結核菌より強毒結核菌の能動的な侵入を反映していることを示唆している。われわれが用いた系は，*in vitro*において結核菌の毒力を解析するために有用であると思われる。

**Key words** : Invasion, *M. tuberculosis*, Non-phagocytic cells, Lung cells, Virulence

キーワーズ：侵入，結核菌，非貪食細胞，肺細胞，毒力

## Introduction

*Mycobacterium tuberculosis* (MTB) is an intracellular pathogen and causes tuberculosis, which remains a major infectious disease in the world. One-third of the world's population is estimated to be infected with MTB[1]. It is widely accepted that infection is established when alveolar macrophages ingest inhaled tubercle bacilli, which survive and replicate in macrophages[2]. However, approximately 90% of infected persons do not develop tuberculosis[3], whereas 10% of infected persons, who are unable to control mycobacterial infection and develop tuberculosis, may have decreased antimicrobial activity of cell-mediated immune system. Thus, establishment of cell-mediated immune system against MTB is crucial for the protective mechanism of host against tuberculosis, in which alveolar macrophages are supposed to play an important role to start cell-mediated immune response against MTB[4].

However, several studies have demonstrated that MTB invades not only macrophages, professional phagocytes, but non-phagocytic cells such as epithelial cells[5)-9)]. It has been also reported that virulent MTB invades more actively than avirulent MTB[8]. In an earlier study, it was suggested that the virulence of MTB observed in experimental animals relates to the ability of MTB to invade and multiply within HeLa cells[10]. More recently, Arruda et al. reported that *Escherichia coli* transfected with a DNA fragment obtained from MTB H37Ra invaded macrophages and HeLa cells more efficiently than non-transfectants[6], suggesting that the genome of H37Ra contains a specific gene that encodes a protein required for invasion of H37Ra into host cells, and that it might be a pathogenicity-related gene of MTB. However, it is likely that tubercle bacilli are phagocytosed by HeLa cells, rather than by invading host cells, because HeLa cells have phagocytic activity[11)12)]. It is also likely that invasion of MTB seen in previous studies was due to phagocytic activity of cell lines used in experiments, because cell lines used in experiments were transformed and may have enhanced phagocytic activity as compared with untransformed cells.

To distinguish the difference between the innate invasive ability of MTB and phagocytosis of host cells, we have determined how many MTB invades non-phagocytic cells. In the present study, we used a human diploid fibroblast cell line, WI-38, and a human epithelial cell line, SQ-5, derived from the lung, to determine the ability of tubercle bacilli to invade and survive within non-phagocytic cells. In addition, we compared the ability of different strains of tubercle bacilli (two virulent strains, MTB Erdman, MTB H37Rv, an attenuated strain MTB H37Ra, a virulent strain *M. bovis* Ravenel, two attenuated *M. bovis* strains, BCG Tokyo and BCG Pasteur, used for vaccination) to invade and survive within non-phagocytic cells.

## Materials and Methods

**① Cell lines and mouse alveolar macrophages**

WI-38 cells, a human diploid fibroblast cell line derived from the normal embryonic lung, and SQ-5 cells, a human epithelial cell line derived from lung squamous cell carcinoma, were obtained from the Riken bank (Wako, Japan). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml of streptomycin, and 100u/ml of penicillin (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan). MH-S, a mouse cell line derived from alveolar macrophage was obtained from ATCC and maintained in PRMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Mouse alveolar macrophages (AMs) were harvested by bronchoalveolar lavage (BAL). Female BALB/c mice were obtained from Charles River, and were between 6 and 10 weeks old. BAL was performed using PBS warmed at 37℃. Cells in BAL fluid were collected by centrifugation at 800 × rpm for 10 min at 4℃, and resuspended at $5 \times 10^5$ cells/ml with MEM containing 10% FCS, 2 mM L-glutamine, 100 μg/ml of streptomycin, and 100u/ml of penicillin.

**② Mycobacteria and growth conditions**

*M. tuberculosis* Erdman (JATA11-03), *M. tuberculosis* H37Rv (KK11-20), *M. tuberculosis* H37 Ra (KK11-05), *M. bovis* Ravenel (KK12-05), *M. bovis* BCG Tokyo (KK12-2) and *M. bovis* BCG Pasteur (JATA12-02) were grown in Middlebrook 7H9 broth (Difco, Detroit, MI, USA) supplemented with ADC (BBL, Cockeysville, MD, USA) and 0.056% of Tween 80. Tubercle bacilli were harvested during the exponential phase of growth, and passed through 5 μm filters to eliminate mycobacterial clumps. Suspension of tubercle bacilli was frozen in 1.0 ml aliquots at -80℃ until use. Bacterial titers of frozen samples were determined by counting colony forming units (CFU) on Ogawa slant medium (Kyokuto, Tokyo, Japan).

**③ Assay for mycobacterial invasion**

WI-38 and SQ-5 cells were inoculated in LabTek chamber slides (Nalge Nunc International, IL, USA), and allowed to grow confluently. Cell numbers of WI-38 and SQ-5 cells at confluent culture were approximately $1 \times 10^8$ cells/ml and $1 \times 10^9$ cells/ml, respectively. Live or heat-killed (autoclaved at 121℃ for 15 min) tubercle bacilli ($1 \times 10^6$ CFU/ml) were added to the confluent cell culture of either WI-38 or SQ-5. Cells were incubated at 37℃ in a 5% $CO_2$ atmosphere for indicated times in antibiotic-free DMEM supplemented with 10% heat-inactivated FCS, and washed three times with antibiotic-free Hanks' balanced salt solution (HBSS) to remove extracellular bacilli from cell culture. Cells were then lysed with 1% Triton X-100 solution, and 10-fold serial dilutions of cell lysates were plated on Ogawa slant medium to count the number of intracellular live bacilli. The number of bacilli was expressed as CFU. For microscopic analyses, cells were fixed and stained by the Ziehl-Neelsen method. Three hundreds cells were observed microscopically and the percentages of infected cells were calculated. Since we used a suspension of single mycobacteria, we did not observe mycobacterial clumps at any time points. For analysis by an electron microscope, cells were fixed with 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde for 2 h. After fixation, cells were stained with 1% $OsO_4$ for 1 h and dehydrated with ethanol. Cells were then embedded in EPON812 (Fluka, Buchs, Switzerland) and were observed electron microscopically using JEM-1200EX (JEOL, Tokyo, Japan).

**④ Assay for phagocytic activity**

Latex beads (3 μM) were purchased from Sigma (St. Louis, MO, USA). WI-38 and SQ-5 cells were inoculated into LabTek chamber slides as described above. Mouse alveolar macrophages ($5 \times 10^5$ cells/ml) and MH-S cells ($1 \times 10^6$ cells/ml) were also inoculated in LabTek chamber slides, and alveolar macrophages were allowed

to adhere to wells for 24 h at 37℃ in a $CO_2$ incubator. Cells were washed once with serum-free MEM to eliminate non-adherent cells. Latex beads were added at 0.005% to cell culture and incubated for 2 h at 37℃ in a $CO_2$ incubator. After incubation, cells were extensively washed three times with serum-free medium to remove unincorporated latex beads. Cells were then fixed and stained to measure incorporated latex beads.

**⑤Multiplication assay for invaded bacilli**

Live bacilli ($1 \times 10^6$ CFU/m*l*) were added to the confluent culture of WI-38 cells. After 2 h. incubation in antibiotic-free DMEM supplemented with 10% heat-inactivated FCS at 37℃, cells were washed three times with antibiotic-free HBSS to remove extracellular MTB from cell culture. Cells were further incubated for 1, 3, and 7 days with antibiotic-free DMEM containing 10% heat-inactivated FCS. After the incubation, serial dilutions of cell lysates were plated on Ogawa slant medium to enumerate CFU.

**⑥Statistical analysis**

The data were analyzed statistically by analysis of variance (ANOVA)[13]. Data represent the mean ± SD of three separate experiments. P values less than 0.05 are considered to be significant.

## Results

**Invasion of tubercle bacilli into human lung cell lines**

To analyze the ability of MTB to invade non-phagocytic host cells, confluent cultures of WI-38 cells and SQ-5 cells were incubated with six strains of tubercle bacilli (Erdman, H37Rv, H37Ra, Ravenel, BCG Tokyo, and BCG Pasteur), which were either live or heat-killed. We first examined the time dependency of MTB invasion into host cells. After various incubation periods, cells were fixed and acid-fast stained, and 300 cells were examined microscopically for the evaluation of mycobacterial invasion. The percentage of infected WI-38 cells in shown in Fig. 1A. Live Erdman and H37Rv more effectively invaded WI-38 when compared to live H37Ra, Ravenel, BCG Tokyo, and BCG Pasteur. The percentages of infected WI-38 cells with live Erdman and H37Rv reached 19.5 ± 4.5% and 22.3 ± 1.0% at 4 h after infection respectively, whereas the percentages of infected cells with H37Ra, Ravenel, BCG Tokyo, and BCG Pasteur at 4 h after infection were 2.0 ± 0.5%, 7.8 ± 1.6%, 8.3 ± 0.4%, and 6.3 ± 0.5%, respectively. Although the percentage of infected cells with Erdman at 4 h after infection was slightly lower than that with H37Rv, Erdman invaded WI-38 cells more rapidly than H37Rv. The time dependency of Ravenel, BCG Tokyo and BCG Pasteur to invade WI-38 cells were similar. Invasion of H37Ra into WI-38 did not significantly increase in a time-dependent manner. Using SQ-5 cells, the similar results were obtained with the exception of H37Ra (Fig. 1B). The percentages of SQ-5 cells infected with live bacilli at 4 h after infection were 48.5 ± 1.5% (Erdman), 55.3 ± 1.8% (H37Rv), 10.7 ± 0.8% (H37Ra), 8.8 ± 0.3% (Ravenel), 10.0 ± 0.4% (BCG Tokyo), and 19.7 ± 0.2% (BCG Pasteur), respectively. The percentages of invasion of live Erdman and H37Rv into SQ-5 cells were 2-fold more than those into WI-38 cells at every time point.

In contrast with live bacilli, numbers of WI-38 cells which incorporated heat-killed bacilli were very low as shown in Fig. 2A (less than 0.5 ± 0.3%), suggesting that WI-38 cells do not have phagocytic activity. On the contrary, SQ-5 cells incorporated intracellularly heat-killed bacilli (Fig. 2B), although the percentages of SQ-5 cells containing heat-killed bacilli were much less than those using live bacilli. These results suggest that live bacilli, especially Erdman and H37Rv, have significant invasive activity.

In order to determine the phagocytic activity of WI-38 and SQ-5 cells, cells were incubated with 3 μm latex beads which are endocytosed mainly by phagocytosis[14]. As shown in Table 1, numbers of incorporated latex beads in SQ-5 cells was approximately 2-fold higher than that in WI-38 cells. To compare the phagocytic activity of these non-phagocytic cell lines, mouse alveolar macrophages and MH-S cells, a mouse

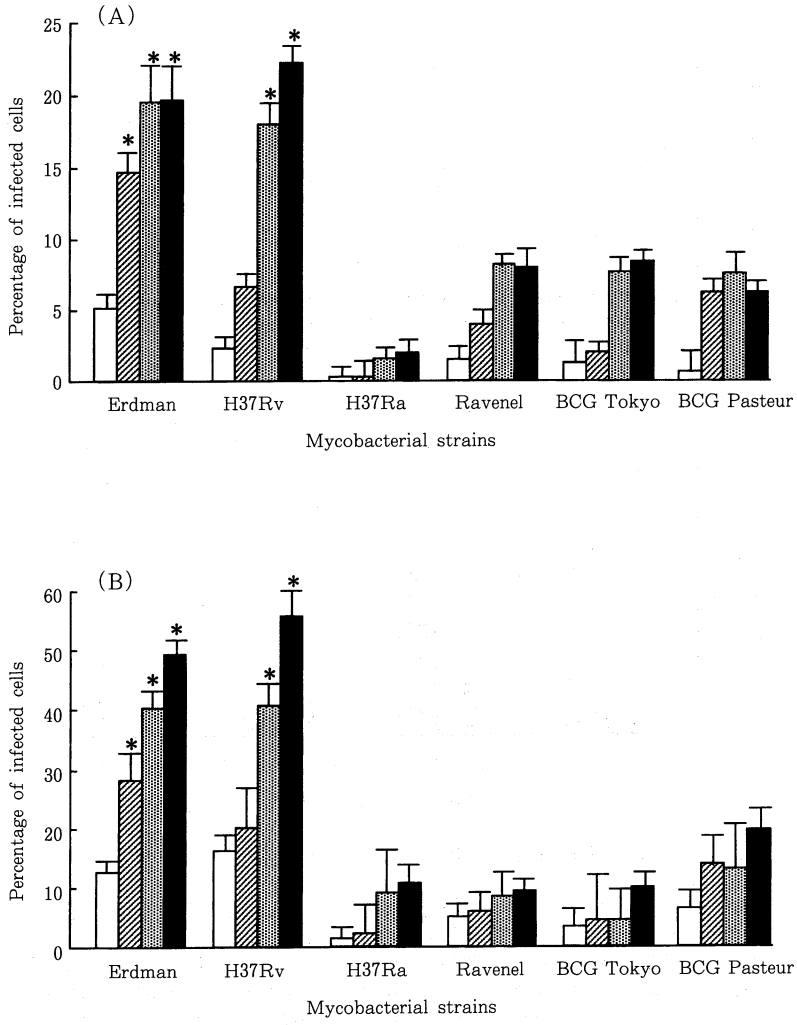


**Fig. 1** Invasion of live tubercle bacilli into WI-38 and SQ-5 cells. WI-38 cells (A) or SQ-5 cells (B) were infected with live *M. tuberculosis* Erdman, H37Rv, H37Ra, *M. bovis* Ravenel, BCG Tokyo and BCG Pasteur for 1 h (□), 2 h (▨), 3 h (▩) and 4 h (■). After infection, cells were washed and stained. Invasion of tubercle bacilli is shown as percentage of infected cells in 300 cells. Data represent mean ± SD of 4 independent experiments. Asterisks indicate a statistically significant difference ($p<0.05$) from numbers of 1 h.

cell line derived from alveolar macrophages, were also incubated with latex beads. Numbers of incorporated latex beads in alveolar macrophages and MH-S cells were much higher than those in WI-38 and SQ-5 cells. Although incorporation of latex beads into WI-38 and SQ-5 were not statistically significant, SQ-5 cells had a tendency to incorporate more latex beads than WI-38 cells.

Fig. 3 shows an electron micrograph of intracellular H37Rv in a WI-38 cell 2 h after infection. The ultrastructural analysis showed the electron-transparent zone, which is a characteristic feature of intracellular mycobacteria [15) 16)],

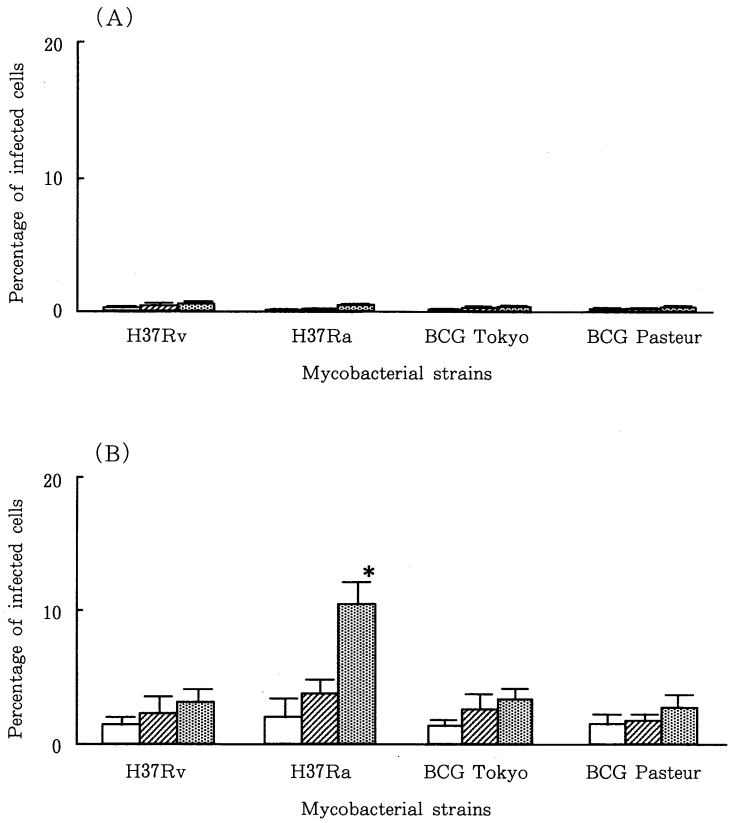


**Fig. 2** Intracellular heat-killed tubercle bacilli within WI-38 and SQ-5 cells.

WI-38 cells (A) or SQ-5 cells (B) were infected with heat-killed *M. tuberculosis* H37Rv, H37Ra, BCG Tokyo and BCG Pasteur for 1h (□), 2h (▨) and 3h (▩). After infection, cells were washed and stained. Invasion of tubercle bacilli is shown as percentage of infected cells in 300 cells. Data represent mean ± SD of 4 independent experiments. An asterisk indicates statistically a significant difference ($p<0.05$) from numbers of 1 h.

**Table 1** Incorporation of latex beads into cells

| Cells | % of latex positive cells |
|---|---|
| WI-38 | 2.4 |
| SQ-5 | 4.3 |
| MH-S | 14.9 |
| Mouse alveolar macrophages | 31.1 |

After stained, 300 cells were counted to calculate the percentages of latex beads incorporating cells (latex positive cells).

and cell-associated bacilli were not observed. These results indicated that bacilli were located inside cells.

**Survival of invaded mycobacteria in WI-38 cells**

To examine the ability of invaded bacilli to survive within host cells, WI-38 cells were infected with live MTB for 2 h. As shown in Teble 2, the number of H37Rv on day 7 increased approximately 94-fold when compared to day 1. Although the number of H37Ra on day 7 also increased compared to day 1 (approximately 6.9-fold), the growth rate of H37Ra was much less than that of H37Rv on day 7. In contrast, the number of BCG Tokyo on day 7 did not increase significantly when compared to day 1 (approximately 1.5-fold). Thus, the growth rate of H37Rv in WI-38 cells was the highest among three strains.

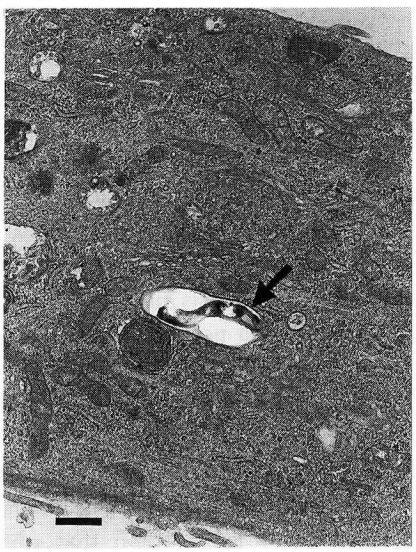

Fig. 3 Transmission electron microphotograph of a *M. tuberculosis*-infected WI-38 cell.
WI-38 cells were infected with *M. tuberculosis* H37Rv at 37℃ for 2h, and then observed by an electron microscope. ×10,000. An arrow indicates *M. tuberculosis* in the cytoplasm of a WI-38 cell. Bar = 500nm.

## Discussion

The virulence of MTB has been assessed by its pathogenicity of tubercle bacilli using experimental animals, such as the survival rate, number and size of lesions, and growth of bacilli in the host. Depending on strains, age and sex of animals used in experiments, the pathogenicity of tubercle bacilli has varied. Thus, the innate virulence of MTB has not been clearly defined. It has been considered that the virulence of MTB consists of both invasion into host cells and survival/multiplication within host cells. Earlier studies have suggested that the innate virulence of MTB correlates with the ability to invade and multiply within HeLa cells[10]. HeLa cells, however, phagocytose pathogenic bacilli by a mechanism similar to that of macrophages[11]. The system cannot distinguish the difference between invaison and phagocytosis. It is, therefore, difficult to determine the innate invasive ability

Table 2 Intracellular multiplication of tubercle bacilli within WI-38 cells

| Bacterial Strains | Cultivation Time | | |
|---|---|---|---|
| | 1 Day | 3 Days | 7 Days |
| H37Rv | $6.4 \pm 0.3 \times 10^5$ | $2.4 \pm 0.2 \times 10^6$ | $6.0 \pm 0.2 \times 10^{7*}$ |
| H37Ra | $1.6 \pm 0.2 \times 10^5$ | $9.6 \pm 0.4 \times 10^5$ | $1.1 \pm 0.1 \times 10^{6*}$ |
| BCG Tokyo | $1.7 \pm 0.1 \times 10^5$ | $3.8 \pm 0.3 \times 10^5$ | $2.6 \pm 0.3 \times 10^5$ |

WI-38 cells were infected with live tubercle bacilli for 2 h at MOI. 10. After removing extracellular bacilli, cells were further incubated for indicated times, and numbers of bacilli were counted as described in Materials and Methods. The results are the means ± SD of four independent experiments. Asterisks indicate a statistically significant difference ($p<0.05$) from numbers of day 1.

of tubercle bacilli using HeLa cells. To distinguish the difference between invasion and phagocytosis, we have used human lung-derived non-phagocytic cells, WI-38 and SQ-5 cells, and assessed invasion and survival of tubercle bacilli within these host cells in the present study.

When WI-38 cells were infected with live tubercle bacilli, two virulent strains, Erdman and H37Rv, efficiently invaded cells, as compared to H37Ra, Ravenel, BCG Tokyo, and BCG Pasteur. The invasion into WI-38 cells by Erdman and H37Rv were observed within 1 h after infection, and this continued throughout the experimental period. Our results that invasive abilities of Erdman and H37Rv to WI-38 cells are similar may be consistent with a recent report that survival curves for Erdman and H37Rv were essentially identical[17]. The invasion of Ravenel, BCG Tokyo, and BCG Pasteur to WI-38 cells were similar, whereas the ability of H37Ra to invade WI-38 cells was much lower than that of Ravenel, BCG Tokyo and BCG Pasteur. Since BCG and H37Ra are similarly attenuated[18], these results suggest that attenuated tubercle bacilli have invasive ability which is considerably low, as compared with virulent bacilli, and that the virulence cannot be determined solely by the ability to invade normal diploid cells.

In contrast to live bacilli, number of heat-killed bacilli inside WI-38 cells was extremely low. These results suggest that number of live Erdman and H37Rv within WI-38 cells reflects active invasion of Erdman and H37Rv regardless of phagocytic activity. Incorporation of latex beads indicates that WI-38 cells do not possess significant phagocytic activity as compared with macrophages. Taken together, mycobacteria within WI-38 cells may represent the invasion but not phagocytosis; therefore, this model may be suitable for analyzing mycobacterial invasion into host cells.

Live Erdman and H37Rv invaded SQ-5 cells, whereas a significant difference was not observed between the invasion rates of other mycobacterial strains into SQ-5. Percentages of phagocytosis of either live or heat-killed BCG Tokyo and H37Ra into SQ-5 cells at 3 h after infection were similar. These results suggest that intracellular mycobacteria at 3 h after infection reflect phagocytosis of SQ-5 cells. Although the amounts of heat-killed H37Rv and BCG Pasteur within SQ-5 cells were similar to that of BCG Tokyo, both live H37Rv and BCG Pasteur efficiently invaded SQ-5 cells in the early stage of infection. Thus, it is suggested that the early invasion of both live H37Rv and BCG Pasteur mainly reflect the active invasion of bacilli rather than phagocytosis of SQ-5 cells. Invasion of virulent tubercle bacilli, Erdman and H37Rv, into SQ-5 cells was approximately three-fold higher than into WI-38 cells. This result suggests that virulent bacilli are quite readily able to invade host cells. Alternatively, the result using SQ-5 cells may reflect both invasion by mycobacteria and phagocytosis of SQ-5 cells, since SQ-5 cells appear to possess higher phagocytic activity than WI-38 cells as shown in the experiment using latex beads. The early incorporation rates of heat-killed H37Rv, H37Ra, BCG Tokyo and BCG Pasteur were similar. However, the amount of heat-killed H37Ra in SQ-5 cells at 3 h after infection was higher than that of heat-killed H37Rv, BCG Tokyo and BCG Pasteur. These results suggest the possibilities that phagocytosis of heat-killed tubercle bacilli at the early stage may be mediated by the common mechanism, and that phagocytosis of heat-killed H37Ra at the late stage may be mediated the additional weak interaction between specific surface molecules of SQ-5 cells and H37Ra. It may be necessary to examine phagocytosis of heat-killed H37Ra into SQ-5 for longer periods.

*M. bovis* Ravenel has been shown to be more virulent than Erdman or H37Rv in mouse systems[19]. However, our results demonstrated that invasive ability of Ravenel into human cells is similar to that of BCG Tokyo and Pasteur, suggesting that virulence of Ravenel may be similar to that of BCG to human.

An electron micrograph of intracellular H37Rv in a WI-38 cell demonstrated the electron-transparent zone, which was suggested to be a defense mechanism of mycobacterial

pathogens[15]. Arruda et al. also showed that tubercle bacilli were surrounded with the electron-transparent zone[6]. Thus, it is likely that the electron-transparent zone which surrounds tubercle bacilli is the common feature in mammalian systems.

H37Rv multiplied most numerously in WI-38 cells when compared to H37Ra and BCG Tokyo. Although numbers of bacilli on day 1 may reflect the invasion, H37Rv multiplied 100-fold on day 7 when compared to day 1. These results suggest that multiplication of bacilli within host cells is an innate feature of virulent mycobacterial strains and that growth rates at the late stage of infection relate to the virulence observed in experimental animals. Taken together, these results suggest that the virulence is based on two phenomena, bacterial invasive ability and multiplication/survival in host cells.

Although MTB is phagocytosed by macrophages through their cell surface receptors for complement[20] and mannose[21], the invasion mechanism of MTB into non-phagocytic cells is not clear. It has been demonstrated that BCG invades cancer cells of the urinary bladder through intercellular adhesion molecules, fibronectin and integrin[22,23]; that *M. leprae* binds to $\beta 1$ integrin of nasal epithelial cells[24]; and that *M. avium-M. intracellulare* binds to the integrin receptor $\alpha_V\beta_3$ on human monocytes[25]. Thus, it is likely that MTB invades non-phagocytic cells through receptors similar to these adhesion molecules. The concept may be supported by the result that H37Rv binds to type II alveolar epithelial cells through vitronectin receptors and $\beta 1$ integrin[8]. In addition, H37Rv may invade non-phagocytic cells through the cytolytic mechanism[26]. Although we have not analyzed binding receptors that may be involved in invasion, the difference between live and heat-killed bacilli in mycobacterial invasion to WI-38 cells suggests that invasion of live MTB into WI-38 cells represents the pathogenic feature of bacilli.

During the process of infection with MTB and disease progression, macrophages[27,28] and neutrophils[29,30] are known to play important roles. However, it is obviously reasonable to assume that inhaled tubercle bacilli first come into contact with bronchial epithelial cells. It is therefore necessary to consider the interaction of tubercle bacilli and non-phagocytic cells. Genetic analysis of *M. tuberculosis* involved in the active invasion is important to understand the relationship between *M. tuberculosis* and host cells.

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