THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 38, Issue of September 17, pp. 39798–39806, 2004
Printed in U.S.A.

Extracellular Mycobacterial DNA-binding Protein 1 Participates in *Mycobacterium*-Lung Epithelial Cell Interaction through Hyaluronic Acid*

Received for publication, March 9, 2004, and in revised form, June 3, 2004
Published, JBC Papers in Press, July 2, 2004, DOI 10.1074/jbc.M402677200

Keiko Aoki‡, Sohkichi Matsumoto‡§, Yukio Hirayama‡, Takayuki Wada‡¶, Yuriko Ozeki‡||, Makoto Niki‡, Pilar Domenech**, Kiyoko Umemori‡‡, Saburo Yamamoto‡‡, Amao Mineda§§, Makoto Matsumoto¶¶, and Kazuo Kobayashi‡

*From the ‡Department of Host Defense, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan, the ¶Osaka City Institute of Public Health and Environmental Sciences, Osaka 545-8585, Japan, ||Osaka International College for Woman, 6-21-57 Tohdacho, Moriguchi, Osaka 570-8555, Japan, the **Tuberculosis Research Section, Laboratory of Immunogenetics, NIAID, National Institutes of Health, Rockville, Maryland 20852, the ‡‡Department of Bacterial and Blood Products, National Institute of Infectious Diseases, Musashimurayama-shi, Tokyo 208-0011, the §§Department of Oral Histology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan, and the ¶¶Otsuka Pharmaceutical Co., Ltd., Kagasuno 463-10 Kawauchi-cho, Tokushima 771-0192, Japan*

***Mycobacterium tuberculosis* infects not only host macrophages but also nonprofessional phagocytes, such as alveolar epithelial cells. Glycosaminoglycans (GAGs) are considered as the component of mycobacterial adherence to epithelial cells. Here we show that extracellularly occurring mycobacterial DNA-binding protein 1 (MDP1) promotes mycobacterial infection to A549 human lung epithelial cells through hyaluronic acid (HA). Both surface plasmon resonance analysis and enzyme-linked immunosorbent assay revealed that MDP1 bound to HA, heparin, and chondroitin sulfate. Utilizing synthetic peptides, we next defined heparin-binding site of 20 amino acids from 31 to 50 of MDP1, which is responsible for the specific DNA-binding site of MDP1. MDP1 bound to A549 cells, and exogenous DNA and HA interfered with the interaction. The binding was also abolished by treatment of A549 cells with hyaluronidase, suggesting that HA participates in the MDP1-A549 cell interaction. Adherence of bacillus Calmette-Guérin (BCG) and *M. tuberculosis* to A549 cells was inhibited by addition of HA, DNA, and anti-MDP1 antibody, showing that MDP1 participates in the interaction between mycobacteria-alveolar epithelial cells. Simultaneous treatment of intratracheal BCG-infected mice with HA reduced the growth of BCG *in vivo*. Taken together, theses results suggest that HA participates in *Mycobacterium*-lung epithelium interaction and has potential for therapeutic and prophylactic interventions in mycobacterial infection.**

Attachment of microbial pathogens to host cells is a critical event to develop mucosal infection (1). *Mycobacterium tuberculosis* persistently infects 32% of the world human population and is responsible for around 1.7 million deaths attributable to a single infectious pathogen each year (2). *M. tuberculosis* is transmitted by airborne particles and is deposited in a terminal alveolus, where the bacteria are phagocytosed by alveolar macrophages or invade into nonprofessional phagocytic cells such as epithelial pneumocytes (3, 4). Nonprofessional phagocytic cells are presumed to be hiding places of *M. tuberculosis* in persistent infection, because *M. tuberculosis* DNA can be frequently detected in type II alveolar epithelial cells and fibroblasts of the lung derived from tuberculin skin test-positive healthy individuals (5). However, the precise mechanism of the interaction between lung epithelial pneumocytes and mycobacteria remains unknown. Better understanding of the interaction is important for developing therapeutic/prophylactic strategies against tuberculosis.

Carbohydrates, such as glycosaminoglycans (GAGs),[1] are thought to be receptors in the process of mycobacterial infection to nonprofessional phagocytic cells (6). GAGs contain hyaluronic acid (HA), heparin, heparan sulfate, and chondroitin sulfates (7). Bacterial pathogens possess adhesion molecule, called adhesin to interact with host tissues. Heparin-binding hemagglutinin (HBHA) binds to GAGs and is identified as a mycobacterial adhesin in the mycobacterium-epithelial cell interaction (6). When bacillus Calmette-Guérin (BCG) or *M. tuberculosis* lacking HBHA was instilled into mice through the intranasal route, delay of extrapulmonary dissemination was observed (8). Thus interaction with epithelia is thought to be crucial for dissemination and to dominate the disease outcome. Besides HBHA, *M. tuberculosis* has another possible adhesin, designated mycobacterial cell entry protein 1 (Mcep1), of which DNA fragmentation confers on *Escherichia coli* an ability to invade into nonphagocytic HeLa cells (9), although its precise role as an adhesin remains unclear (10). It is likely that myco-

* This work was supported by grants from the Ministry of Health, Labour and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants), The Japan Health Sciences Foundation, Ministry of Education Culture Sports Science and Technology, Inamori Foundation, Osaka City University (Urban Research Project), and The United States-Japan Cooperative Medical Science Program against Tuberculosis and Leprosy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 81-6-6645-3746; Fax: 81-6-6645-3747; E-mail: sohkichi@med.osaka-cu.ac.jp.

[1] The abbreviations used are: GAG, glycosaminoglycan; aa, amino acid; BCG, bacillus Calmette-Guérin; BSA, bovine serum albumin; fluos, 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester; HA, hyaluronic acid; HBHA, heparin-binding hemagglutinin; GFP, green fluorescent protein; LBP, laminin-binding protein; MDP1, mycobacterial DNA-binding protein 1; P31–50, synthetic peptide corresponding to the amino acid sequence of MDP1 at the 31–50 position; RU, resonance units; SPR, *surface plasmon resonance*; CBB, Coomassie Brilliant Blue; CHO, Chinese hamster ovary cells; CFU, colony-forming units.

This paper is available on line at http://www.jbc.org

bacteria utilize multiple adhesins to promote attachment to nonprofessional phagocytes.

A number of nonspecific DNA-binding proteins are found in association with bacterial chromosomes and are called histone-like proteins. Mycobacterial DNA-binding protein 1 (MDP1) has a wide range of binding activities to genomic DNA through guanine and cytosine and is an abundant structural protein, which constitutes ~7% of cellular proteins in BCG and *M. tuberculosis* H37Rv (11). MDP1 possesses a DNA-binding site at the position of amino acids (aa) 31–50, the sequence of which does not exist in any other nucleic acid-binding protein (12). MDP1 has partial homology with HU, histone-like protein of *E. coli*, in the NH_2-terminal region and with eukaryotic histone H1 in the COOH-terminal region. These facts indicate that MDP1 is mycobacteria-specific histone-like protein (11). Importantly, MDP1 inhibits macromolecular biosyntheses of DNA, RNA, and protein *in vitro* and recombinant *E. coli* expressing MDP1 grows much slower when compared to the parental strain (13). The expression of MDP1 is up-regulated at the stationary or dormant phases of mycobacterial culture (11, 14). MDP1 and its homologues, which have been designated as histone-like protein (14) and laminin-binding protein (LBP) (15), are distributed in all mycobacterial species so far examined (12). These imply that MDP1 plays an important role in both slow growth and dormant/latent infection with mycobacteria.

Another interesting feature of this molecule is localization. MDP1 exits not only in the cytoplasmic space associated with the 50 S ribosomal subunit and presumably nucleoid but also on the bacterial surface (11, 15). Nuclear protein is localized in the cytoplasm, and its primitive roles are to compact the genome and control gene expression. However, some nuclear proteins are expressed on the cell surface and play alternative roles. For example, eukaryotic histone H1 on the cell membrane is identified as a receptor for thyroglobulin that is the precursor of thyroid hormones (16). High mobility group 1 protein is a eukaryotic nuclear protein associated with chromatin but also secreted in the extracellular milieu (17). Outside the cell, high mobility group 1 binds to the receptor for advanced glycation end products and then stimulates the cell damage signal. This signal eventually triggers inflammation for clearance of necrotic cells (18–20). *Mycobacterium leprae* produces LBP, which is a homologue of MDP1, although it lacks DNA-binding activity (15). Instead of DNA-binding activity, LBP interacts with laminin-2 and is considered to be involved in *M. leprae* invasion into Schwann cells of the peripheral nervous system (15).

The aim of this study was to clarify the role of surface-exposed MDP1 in the interaction between mycobacteria and lung epithelial cells, because DNA-binding proteins tend to bind GAGs that are thought to be receptors in the process of mycobacterial infection to nonprofessional phagocytes (6, 7). Our results demonstrate that extracellular MDP1 acts as an adhesin by binding to GAGs and mediates mycobacterial adherence to A549 human lung epithelial cells by interaction with hyaluronic acid (HA). Treatment of BCG-infected mice with HA resulted in the reduction of mycobacterial growth *in vivo*. Taken together, HA may play a key role in adherence of mycobacteria to lung epithelial cells.

EXPERIMENTAL PROCEDURES

Culture Medium and Reagents—RPMI 1640 media, L-glutamine, and 0.05% trypsin EDTA solution, heparin from porcine intestinal mucosa, HA from human umbilical cord, and heparan sulfate from bovine kidney were purchased from Sigma. Chondroitin sulfate A and C were purchased from Calbiochem. Hyaluronidase from *Streptomyces hyalurolytics*, heparinase 1 from *Flavobacterium heparinum*, and chondroitinase ABC from *Proteus vulgaris* were purchased from Sigma. Polyclonal anti-MDP1 sera were obtained from female rabbit (Japan SLC, Shizuoka, Japan) after multiple injections of MDP1 emulsified in incomplete Freund's adjuvant. Polyclonal anti-MDP1 antibodies were obtained by 30% ammonium sulfate precipitation of antisera. The salt was then removed by dialysis in PBS.

Preparation of Subcellular Fractions of BCG—To obtain subcellular fractions from BCG, all the following procedures were carried out at 4 °C. Ten grams of BCG was disrupted in cold PBS with a Bioruptor UCD-200T sonicator (Toso, Tokyo, Japan), and the suspension was centrifuged at 3,000 × *g* for 5 min to remove unbroken bacteria. The supernatant was centrifuged at 10,000 × *g* for 10 min. The pellet was rinsed with cold PBS and again centrifuged at 10,000 × *g* for 10 min. This cell wall-containing pellet was resuspended in 4 ml of cold PBS, and then Percoll (Amersham Biosciences) was added to be 60%. Next the mixture was centrifuged at 27,000 × *g* for 1 h to separate cell walls from unbroken cells. The cell wall band was collected and washed twice with PBS and was used as the cell wall fraction. A membrane-ribosome fraction was obtained by centrifugation of the supernatant of the cell wall-containing pellet at 105,000 × *g* for 2 h. The pellet and the supernatant were used as membrane-ribosome and cytoplasmic fractions, respectively. Culture supernatants of BCG were obtained filtration of culture media of BCG through the membrane filter (pore size of 0.22 μm) and concentrated by 80% of ammonium sulfate precipitation. The salt was then removed by dialysis in PBS. Twenty micrograms of each fraction was fractionated by SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and reacted with polyclonal anti-MDP1 antisera.

Immunogold Electron Microscopy—BCG and *Mycobacterium smegmatis* were grown in Middlebrook 7H9-ADC media at 37 °C until $A_{1.5}$, and then the bacteria were collected by centrifugation. The bacterial pellet was fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer, pH 7.4, for 2 h at 4 °C and post-fixed in 1% osmium tetraoxide in the same buffer for 1 h at 4 °C. Specimens were dehydrated in graded ethanol and embedded in Epon-Araldite resin. The thin section was mounted on the nickel grids, treated in 3% H_2O_2 for 10 min, and washed in water. For blocking the nonspecific binding of antibody to the plastic, the section was preincubated with 3% BSA in PBS for 60 min at room temperature and washed by PBS, followed by incubation with rabbit anti-MDP1 antisera diluted in PBS containing 0.1% BSA and 0.05% Tween 20 (PBS-B-T, 1:800) for 2 h at room temperature. The section was then rinsed for 30 min and incubated with protein A conjugated with gold of 10 nm in diameter (EY Laboratories Inc., San Mateo, CA) diluted in PBS-B-T (1:100) for 1 h at room temperature. Finally, the section was rinsed with PBS and distilled water and then briefly stained with uranyl acetate and Reynolds' lead citrate. The section was analyzed using an H7100 electron microscope (Hitachi Co., Ltd., Tokyo, Japan) operated at 75 kV.

Proteins Purification—Native MDP1 and antigen 85B were purified from BCG strain Tokyo as described previously (11, 21). Recombinant MDP1 and HBHA were obtained as follows. Based on the nucleotide sequences of *mdp1* and *hbha*, the oligonucleotide primers, *i.e.* the forward (5′-CCCCATATGAACAAAGCAGAGCTCATTGAC-3′ and 5′-CATATGGCTGAAAACTCGAACAT-3′) and the reverse (5′-CCCAAGCTTTTTGCGACCCCGCCGAGCGG-3′ and 5′-AAGCTTCTGGGTGACCTTCTTG-3′) primers, were synthesized, respectively. The PCR was carried out by targeting 10 ng of chromosomal DNA derived from BCG in an automated thermal sequencer (Iwaki Glass Co., Tokyo, Japan). The samples were first denatured by heating at 94 °C for 5 min and then incubated for 30 cycles at 94 °C for 2 min, 56 °C for 80 s, and 72 °C for 5 min and then finally incubated for 5 min at 72 °C. The amplified DNA fragment, including *mdp1* and *hbha*, were digested by NdeI and HindIII, and inserted into the same sites of pET22b+ (Novagen, Darmstadt, Germany). These plasmids were designated pET22b-MDP1 and pET22b-HBHA, respectively. The DNA sequences of the cloned genes were confirmed by using an ABI 373 automatic DNA sequencer (Applied Biosystems, Foster, CA). The pET22b-MDP1 and pET22b-HBHA were introduced into *E. coli* BL21(DE3)pLys (Invitrogen), and these recombinant bacteria were harbored in LB media containing 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol at 22 °C. When an optical density at 600 nm of the culture reached 0.3–0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.1 mM, and then the mixture was incubated for additional 7 h. The cells were sonicated as described above, and the supernatant was collected after centrifuging 8,000 × *g* for 30 min. After removing the clamp from the supernatant by filtration through a 0.22-μm membrane filter, it was applied to a 1-ml Hi-Trap chelating column previously charged with 100 mM $NiSO_4$ and equilibrated with 20 mM sodium phosphate, pH 7.4, 10 mM imidazole, and 0.5 M NaCl. After unbound proteins were washed

out, the protein was eluted with the same buffer containing 300 mM imidazole. The fractions containing MDP1 were collected and dialyzed against PBS. The purity of proteins was confirmed by SDS-PAGE analysis and stained with Coomassie Brilliant Blue R-250 (CBB) as a single band.

Heparin-Sepharose Chromatography—An MDP1-rich, acid-soluble protein fraction was obtained from BCG as described before (11). 200 μg of acid-soluble protein was fractionated by heparin-Sepharose chromatography (Amersham Biosciences), of which bed volume was 1 ml, at room temperature with a linear gradient of NaCl in PBS. Gradients were made in a gradient apparatus filled with 10 ml each of 0.15 and 2 M NaCl solution. A flow rate of 1 ml/min was maintained, and 1 ml of each fraction was collected. The fraction was analyzed by SDS-PAGE. The protein bands were stained with CBB.

Surface Plasmon Resonance Measurements—The interaction between MDP1 and GAGs was monitored by measuring SPR using a BIAcore 2000 biosensor (BIAcore AB, Uppsala, Sweden). All binding reactions were performed at 25 °C in 10 mM HEPES buffer, pH 7.4, including 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20. Proteins and peptide were immobilized to the dextran matrix on the CM5 sensor chip (BIAcore) using an amine coupling kit according to the manufacturer's instructions (BIAcore). Association and dissociation rate constants were calculated by nonlinear fitting of the primary sensorgram data using BIAevaluation software version 3.0.

Determination of the Heparin-binding site by ELISA—20-mer of synthetic sequential peptides corresponding to the amino acid sequence of MDP1 (12) were immobilized on the 96-well ELISA plate (Sumitomo, Osaka, Japan) at a concentration of 10 μg/ml in carbonate buffer, pH 9.6, at 4 °C overnight. After blocking the wells by 5% BSA in PBS, biotinylated heparin (Sigma) was added at 1 μg/ml in PBS containing 0.05% Tween 20 (PBS-T) to each well and incubated for 1 h at 37 °C. After washing unbound heparin, horseradish peroxidase-conjugated streptavidin was added and the mixture was incubated for 1 h at 37 °C. After washing free streptavidin, binding was detected by color development with *o*-phenylendiamine dihydrochloride (Wako, Tokyo, Japan), and ELISA units (optical density) were measured at 492 nm.

Inhibition of interaction between MDP1 and heparin by the synthetic peptide was examined by the following procedure. MDP1 was immobilized on the 96-well ELISA plate at a concentration of 4 μg/ml in carbonate buffer, pH 9.6, at 4 °C overnight. Biotinylated heparin and peptide were premixed in PBS-T for 10 min at 37 °C and added into each MDP1-immobilized well. After 30-min incubation at 37 °C, unbound heparin was washed out and the level of binding was determined by horseradish peroxidase conjugated with streptavidin as described above.

Detection of MDP1 Binding to GAGs by ELISA and Inhibition Assay by Exogenous DNA—Genomic DNA of BCG and HA were immobilized on the 96-well ELISA plate at 10 μg/ml and heparin and chondroitin sulfate C were done at 100 μg/ml overnight incubation at 37 °C. MDP1 at 1 μg/ml in PBS-T was added into wells and incubated for 1 h at 37 °C. Wells were washed, and then anti-MDP1 antisera diluted by PBS-T to 1 to 1000 were added. After 1-h incubation at 37 °C, wells were washed and peroxidase-conjugated anti-rabbit antibody was added. After washing the wells, MDP1 binding was detected by color development with *o*-phenylendiamine dihydrochloride as described above.

Protein Labeling—MDP1 was labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (fluos, Roche Diagnositics GmbH, Mannheim, Germany). One milligram of MDP1 was mixed with 0.237 mg of fluos in 1 ml of PBS for 2 h at room temperature. The molar ratio of fluos to MDP1 is 10 to 1. Then, nonreacted fluos was separated by gel filtration on the Sephadex G-25 column (Amersham Biosciences). Similarly, bovine serum albumin (BSA, Sigma) and HBHA were labeled with fluos (molar ratio, 10 to 1). Concentrations of labeled proteins were determined by Bradford's method (22) using BSA as a standard.

Protein Binding Assay to A549 Type II Human Lung Epithelial Cells—A549 cells were suspended at 2×10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum (Sigma), 25 mM HEPES, 2 mM L-glutamine, 5.5×10^{-5} M 2-mercaptoethanol (complete culture medium), and 1 ml of cell suspensions was dispensed into individual wells of a 24-well plate (BD Biosciences). Plates were incubated at 37 °C in a humidified atmosphere at 5% CO_2 for 24 h. The nonadherent cells were poured off, and the residual nonadherent cells were removed by washing with serum-free RPMI 1640 medium twice and refilled with 200 μl of complete culture medium. fluos-labeled MDP1, BSA, or HBHA was added to a final concentration of 0.5 μM, and the mixture was incubated for 0–5 h. In experiments of enzymatic treatment, epithelial cells were treated with heparinase 1, hyaluronidase, or chondroitinase ABC (Sigma) before incubation with proteins. One unit of each enzyme was added into each well and incubated for 2 h in PBS at 37 °C under 5% CO_2. Then enzymes were removed by washing with PBS twice and incubated with fluos-labeled proteins. After the incubation, wells were washed three times with RPMI 1640 medium at 37 °C to remove nonadherent proteins. Then cells were detached by using cell scrapers and collected in a tube and centrifuged at 300 × *g* for 5 min. After removing supernatant, cells were fixed by adding 1 ml of 1% paraformaldehyde-PBS. Fixed cells were analyzed by flow cytometry using Cellquest™ software (BD Biosciences).

Construction of Mycobacteria Expressing Green Fluorescent Protein—A BamHI-EcoRI 0.7-kbp fragment from the vector pEGFP (BD Biosciences) containing the gene encoding GFP was introduced into the *E. coli*-mycobacteria shuttle vector pMV261 (23) to generate the plasmid pMV261GFP. This plasmid was introduced into BCG by electroporation, and kanamycin-resistant BCG colonies were selected after 3 weeks of culture on Middlebrook 7H11 agar containing oleic acid, dextrose, albumin, and catalase enrichment (Difco, 7H11-OADC agar) in the presence of 20 μg/ml kanamycin. The expression of GFP was confirmed by confocal laser microscopy LSM510 (Carl Zeiss, Tokyo, Japan) according to the manufacturer's instructions.

Experimental Infection in Vitro—Mycobacterial suspension was prepared by the conventional method (24) after harvesting bacteria in 7H9 media (Difco) supplemented with 10% albumin, dextrose, and catalase enrichment (Difco) and 0.05% Tween 80 at 37 °C until 0.6 of optical density at 650 nm. Bacterial suspensions were added to A549 epithelial cells at multiplicities of infection ranging from 1 to 10 bacteria/epithelial cell. After either 6- or 24-h incubation, unbound bacteria were washed with RPMI 1640 medium three times, and adherent epithelial cells were collected by scraping with rubber policeman. Serial 10-fold dilutions of the cell suspension were cultivated on 7H11-OADC agar. Colonies were counted after 3 weeks incubation at 37 °C. Cells infected with BCG expressing GFP were assessed by flow cytometry (FACScan, BD Biosciences) as described in the fluos-labeled protein binding assay.

Experimental Infection in Vivo—Female C57BL/6 mice, 7 weeks of age (Japan Clea, Suita, Osaka, Japan), were challenged intratracheally with 1×10^6 CFUs of BCG in the presence or absence of 50 μg of HA. At 2 weeks of infection, lungs of mice were removed aseptically and homogenized individually with a set of motor and pestle. The homogenate was plated on 7H11-OADC agars after 10-fold serial dilution. Mycobacterial colonies were counted 3 weeks after the culture.

Statistical Analyses—Data were analyzed by Power Macintosh G4 using StatView 5.0 (SAS Institute Inc., Cary, NC) and expressed as the mean ± S.D. Data that appeared statistically significant were compared by an analysis of variance for comparing the means of multiple groups and considered significant if p values were less than 0.05.

RESULTS

Cellular Localization of MDP1—We examined cellular localization of MDP1 by both biochemical and ultrastructural analyses. Subcellular fractions obtained from BCG were examined by Western blot analysis by anti-MDP1 antisera (Fig. 1*A*). The result showed anti-MDP1 antibody reacted with the bands in the cell wall and the membrane-ribosome fractions. In contrast, antibody failed to react with cytoplasmic fraction and culture filtrates. Next immunogold electron microscopic examination was carried out. Protein A-coupled with gold particles did not react with the section (Fig. 1*B*). In contrast, anti-MDP1 antibody reacted with both inside and outside mycobacteria (Fig. 1*C*). These results indicate that MDP1 localizes on/in the cell wall as well as intracellular ribosome and membrane fractions. This agrees with previous findings (11, 15).

Amino Acids Sequence Homology between MDP1 and HBHA—Sequence alignments among BCG-MDP1, *M. tuberculosis*-MDP1, and *M. tuberculosis*-HBHA were carried out by a malign program through the DNA data base of Japan (Fig. 2). BCG-MDP1 revealed a lack of nine amino acids from *M. tuberculosis*-MDP1, and the identity of amino acid sequences was 95%. The total sequence homology between MDP1 and HBHA was low (BCG-MDP1 and HBHA, 35%; *M. tuberculosis*-MDP1 and HBHA, 34%), although both proteins possessed the conserved region in the C-terminal (Fig. 2). HBHA possessed a heparin-binding site in the C-terminal region, and four PAKK repetitive sequences are key residues for binding to GAGs (25,

26). Six PAKK repetitive sequences were seen in the C-terminal region of MDP1 (Fig. 2).

MDP1 Binds to Heparin—To explore the role of extracellularly occurring MDP1, we examined the binding capacity of MDP1 to heparin. Acid-soluble proteins enriched MDP1 derived from BCG (11) were applied on heparin-Sepharose chromatography, and heparin-binding proteins were fractionated by chromatography with a linear gradient from 0.15 to 2 M NaCl (Fig. 3A). Major 28-kDa protein on SDS-PAGE analysis was eluted by the fraction peak at 11 that was equivalent to 1 M NaCl. This protein was confirmed as MDP1 by Western blotting analysis utilizing monoclonal antibody (data not shown) and by determining the N-terminal amino acid sequences. It was reported that HABA was eluted at around 0.3 M NaCl by heparin-Sepharose column chromatography (26). Thus MDP1 is likely to bind to heparin with stronger affinity comparing to HBHA.

A
kDa 1 2 3 4 5
94 —
67 —
43 —
30 —
20.1—

B C

FIG. 1. **Cellular localization of MDP1 in mycobacteria.** *A*, subcellular localization of MDP1 analyzed by the Western blot. Subcellular fractions were reacted with anti-MDP1 antisera on the transferred membrane after fractionated by SDS-PAGE. *Lanes*: *1*, cell lysate of BCG; *2*, cell wall fraction; *3*, membrane-ribosome fraction; *4*, cytoplasmic fraction; *5*, culture filtrate. *B* and *C*, immunogold electron microscopic examination. *B*, control, pre-immune rabbit sera reaction. *C*, anti-MDP1 antisera reaction. Magnification factor is ×20,000. *Bar*, 1 μm.

SPR Analysis of the Interaction between MDP1 and GAGs—To clarify the molecular interaction between MDP1 and GAGs, SPR analysis was performed. In the preliminary experiment, we found that MDP1 bound to both basic sensor chip C1 and dextran-coated sensor chip CM5. Based on this result, we subjected MDP1 as an immobilized ligand in the subsequent experiments.

Native MDP1 and antigen 85B, a 28-kDa fibronectin-binding protein (27, 28), were immobilized on the CM5 sensor chip until gaining 3332 and 3312 resonance units (RUs), respectively. Heparin (10 μg/ml), as an analyte, was injected into the control sensor cell and immobilized cells with either MDP1 or antigen 85B for 20 s. As expected from the result of heparin-Sepharose column chromatography analysis, the interaction between MDP1 and heparin was observed until gaining 300 RU. Heparin did not interact with antigen 85B and empty sensor.

To know whether MDP1 binds to possible receptors for mycobacteria in adherence to epithelial cells, we extended the study to other GAGs. It was previously reported that, in addition to heparin, adherence to Chinese hamster ovary (CHO) cells was inhibited by chondroitin sulfate, but not dextran, mannose, and galactose (6). Accordingly we examined these carbohydrates by SPR analysis. The results showed that chondroitin sulfate A, and chondroitin sulfate C bound to MDP1. The gaining maximum RUs were 63 and 55, respectively. In contrast, other carbohydrates such as dextrose, mannose, galactose, arabinose, and trehalose did not bind to MDP1 (data not shown). These data show that MDP1 binds to certain GAGs that are possible receptors for adherence to CHO cells (6). Also, MDP1 bound to HA and heparan sulfate with 90 and 89 RU, respectively (data not shown). All carbohydrates tested failed to bind to empty sensor or antigen 85B immobilized sensor cells.

Next we attempted to determine the affinity between MDP1 and GAGs. Because of the limitation of employing GAGs of certain molecular size, affinities were determined for heparin (M_r 5,000, Fig. 3*B*) and heparan sulfate (M_r 10,000, Fig. 3*C*) (Table I). Because true binding affinity cannot be determined for the ligand with multivalent, pseudo apparent rate constants (k_a and k_d) were calculated by the basic model. The affinity (K_D) of heparin and heparan sulfate was 3.53×10^{-10} and 1.54×10^{-8}, respectively.

Previously, real-time association between HBHA and heparin was studied by SPR analysis (26). In comparison to that, MDP1 associates with heparin more quickly and dissociates more slowly (HBHA to heparin, k_a: 5.62×10^5 M^{-1} s^{-1}, k_d:

```
BCG-MDP1   MNK-------------------------AELIDVLTQKLGSDRRQATAAVENVVDTIVRA    35
Mtb-MDP1   MNK-------------------------AELIDVLTQKLGSDRRQATAAVENVVDTIVRA    35
Mtb-HBHA   MAENSNIDDIKAPLLAALGAADLALATVNELITNLRERAEETRTDTRSRVEESRARLTKL    60
           *                           ***  *         *      **

BCG-MDP1   VHKGDSVTITGFGVFEQRRRAARVARNPRTGETVKVKPTSVPAFRPGAQFKAVVSGAQRL    95
Mtb-MDP1   VHKGDSVTITGFGVFEQRRRAARVARNPRTGETVKVKPTSVPAFRPGAQFKAVVSGAQRL    95
Mtb-HBHA   QEDLPEQLTELREKFTAEELRKAAEGYLEAATSRYNELVERGEAALERLRSQQSFEEVSA   120
                         *

BCG-MDP1   PAEGPAVKRGVGASAAKKVAKKAPAKKATKAAK---------KAATKAPAKKAATKAPAK   146
Mtb-MDP1   PAEGPAVKRGVGASAAKKVAKKAPAKKATKAAKKAATKAPARKAATKAPAKKAATKAPAK   155
Mtb-HBHA   RAEGYVDQAVELTQEALGTVASQTRAVGERAAK---------LVGIELPKKAAPAKKAA-   170
            ***         *              ***                   * * *  *  *

BCG-MDP1   KAVKATKSPAKKVTKAVKKTAVKASVRKAATKAPAKKAAAKRPATKAPAKKATARRGRK   205
Mtb-MDP1   KAVKATKSPAKKVTKAVKKTAVKASVRKAATKAPAKKAAAKRPATKAPAKKATARRGRK   214
Mtb-HBHA   --------PAKKAA---------------PAKKAAAKKAPAKKAAAKKVTQ--------K   199
                   ****                  * ** **** **  * *              *
```

FIG. 2. **MDP1 and HBHA possess PAKK repetitive sequences in the C-terminal regions.** The alignment of amino acid sequences allowing gaps (−) between MDP1 (BCG-MDP and *M. tuberculosis*-MDP1) and HBHA. The alignment was performed by malign program through DDBJ DNA data base. *Asterisks* indicate conserved aa among three proteins. *Blue* and *underlined* sequences represent the MDP1-specific DNA binding region previously determined (8). Repetitive aa sequences, PAKK, are highlighted by *red*. *Mtb*, *M. tuberculosis*.

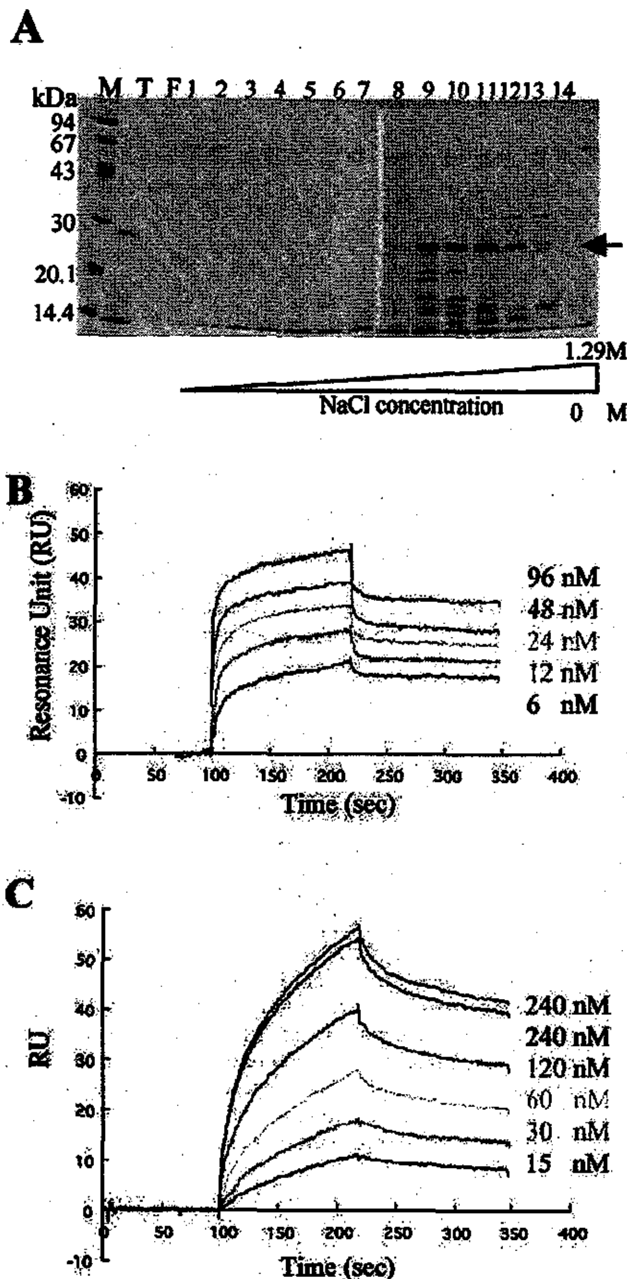

FIG. 3. **MDP1 binds to GAGs.** *A*, size fractionation of elutriates from heparin-Sepharose chromatography by SDS-PAGE. The gel was stained with CBB. *M*, molecular weight markers. *T*, total acid-soluble proteins applied to chromatography. *F*, unbound flow-through fraction. The *arrow* indicates MDP1. *B* and *C*, surface plasmon resonance analysis of the interaction between MDP1 and GAGs. Results for heparin (*B*) and heparan sulfate (*C*) are shown. Each GAG was injected at different concentrations as indicated in the figures and flowed over a surface chip at flow rate of 10 μl/min. RU values are the subtracted values of empty sensor cells from MDP1 immobilized sensor cells.

1.5×10^{-2}, K_D: 2.6×10^{8}). The results of the heparin column study (Fig. 3*A*) and SPR analysis (Fig. 3*B*) indicate that MDP1 possesses much higher affinity to heparin than HBHA.

Determination of the Heparin-binding Site of MDP1—The difference of affinity between MDP1 and HBHA may be explained by binding sites other than PAKK repetitive regions in MDP1. To determine the binding site, we examined the heparin-binding activity of 20-mer of synthetic peptides covering the entire sequence of MDP1 (12). Wells of microtiter plates were coated with synthetic peptides and reacted with biotin-labeled heparin. Heparin bound to the peptide corresponding to an amino acid sequence of MDP1 at the 31–50 position (P31–50) (Fig. 4).

TABLE I
Kinetics parameters for MDP1-GAG interaction from SPR analysis

Immobilized ligand	Analytes	Association rate, k_a	Dissociation rate, k_d	Affinity, K_D
		1/ms	*1/s*	*M*
MDP1	Heparin	1.08×10^{6}	3.83×10^{-4}	3.53×10^{-10}
	Heparan sulfate	9.81×10^{4}	1.51×10^{-3}	1.54×10^{-8}

To exclude errors due to variation of the level of immobilization among peptides, inhibition assay was carried out. Only P31–50 inhibited the binding of heparin to MDP1 (molar ratio, heparin/peptide = 1/100, data not shown). On the contrary, the peptides containing PAKK did not interfere with the interaction between MDP1 and heparin. But this is not controversial, because longer amino acid sequences involving more than three PAKK sequences is required for binding of HBHA to heparin (26). Next we tried to determine the affinity between P31–50 and heparin by SPR analysis. P31–50 bound to both CM5 and C1 sensor chips as MDP1 did. Therefore, P31–50 was subjected to the ligand and immobilized on the CM5 sensor chip until gaining 3015 RU. Injection of heparin (30 s, 10 μg/ml) into P31–50-immobilized cells gained 91 RU, confirming binding activity of P31–50 to heparin, although we could not determine the K_D value by using the BIAevaluation software. For this reason, a large part of the binding site of synthetic peptide may be inactivated and/or masked during immobilization procedure.

Exogenous DNA Inhibits MDP1-GAG Interaction—To provide direct evidence that MDP1 shares DNA- and GAG-binding sites, an inhibition assay was carried out by ELISA. As expected from SPR analysis, binding of MDP1 to GAG was detected as shown in Fig. 5. Binding of MDP1 to GAGs was *inhibited* by exogenous DNA, similar to the inhibition of MDP1-DNA interaction by DNA (Fig. 5). These results have confirmed the binding activity of MDP1 to GAGs and also showed that MDP1 interacts with GAGs by its DNA-binding site.

MDP1 Binds to A549 Human Lung Epithelial Cells—To know whether MDP1 binds to epithelial cells, A549 cells were incubated with fluos-labeled MDP1, fluos alone, and fluos-labeled BSA served as controls. Confocal laser microscopic analysis showed MDP1 (Fig. 6*A*), but not fluos alone and BSA (data not shown), attached to cells. The kinetics and magnitude of the binding were monitored after adding a final concentration of 0.5 μM MDP1 or HBHA by using flow cytometry (Fig. 6*B*). Binding of MDP1 to A549 cells were seen immediately after the addition of MDP1, conspicuous by 10 min, and reached a plateau by 60 min after the addition. More than 95% of A549 cells were MDP1-positive 60 min after. By contrast, binding of HBHA was delayed and more than 10% of cells were HBHA-negative even at the plateau.

To know whether MDP1 binding to A549 cell is dependent on GAGs, the following experiments were performed. First we examined the inhibitory effects of exogenously added GAGs. Addition of either heparin or HA resulted in a dose-dependent and remarkable inhibition of binding, whereas mannose did not (Fig. 6*C*). In contrast, heparin and HA partially inhibited interaction between HBHA and A549 cell, and thus the level of interference was much less comparing to MDP1 (Fig. 6*D*). Next, we determined which GAG actually participates in MDP1 binding to A549 cells utilizing enzymes degrading GAGs. Heparinase 1 degrades heparin, heparin sulfate, and HA (29), and chondroitinase ABC degrades chondroitin sulfate and HA (30). Hyaluronidase derived from *Streptomyces* is specific for HA unlike other hyaluronidases (31). Treatment of

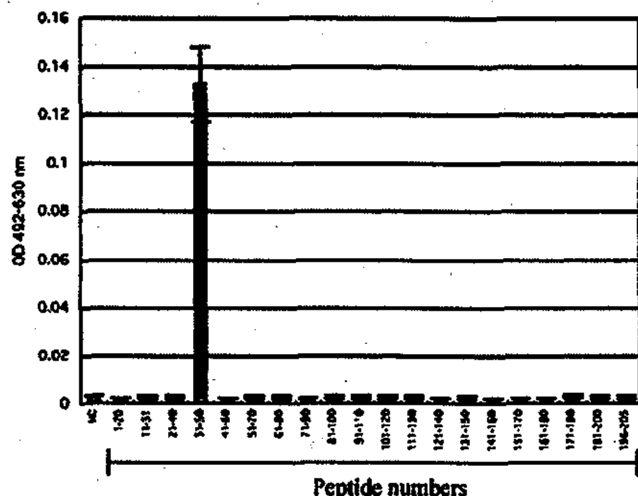

FIG. 4. **The MDP1-specific DNA binding region is the heparin-binding site.** Each peptide corresponding to the amino acid sequence of MDP1 was immobilized, and then biotinylated heparin was reacted. *OD*, optical density; *NC*, negative control without peptide.

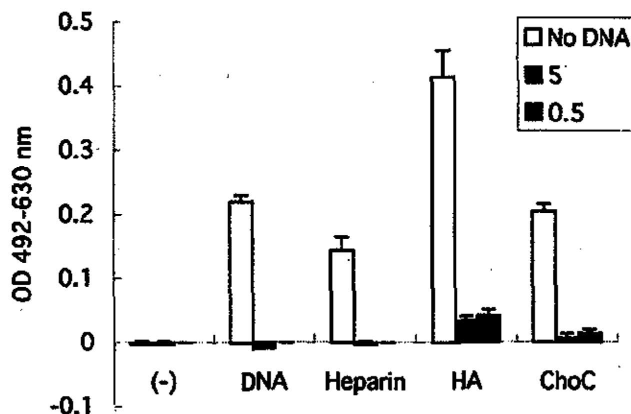

FIG. 5. **DNA interferes with MDP1-GAG interaction.** The wells of ELISA plates were coated with DNA and GAGs, and then MDP1 was added in the presence or absence of exogenous DNA. The level of MDP1 binding was determined by ELISA utilizing anti-MDP1 antisera. *ChoC*, chondroitin sulfate C. *No DNA*, without exogenous DNA. 5 or 0.5 μg of DNA was added to the wells as inhibitors.

A549 cells with these enzymes resulted in a reduction of the binding (untreated, 93.0%; treated with heparinase 1, 38.5%; chondroitinase ABC, 26.8%; hyaluronidase, 5.8%; and the combination of enzymes, 3.7%) (Fig. 7.) These results indicate that MDP1 binds to HA on A549 cells.

Inhibition of MDP1-A549 Cell Interaction by Exogenous DNA—We have already demonstrated that the DNA-binding region of MDP1 interacts with heparin (Figs. 4 and 5). The result prompted us to examine the possibility that the region may participate in the binding to A549 epithelial cells. The addition of exogenous DNA inhibited the binding of MDP1 (Fig. 8), but not HBHA (data not shown), to A549 cells. Based on the fact that MDP1 and its fragment, P31–50, bound to DNA via guanine and cytosine (11), we examined the effect of oligonucleotide DNA with 20-mer of dideoxyguanine (poly(dG)) on the interaction between MDP1 and A549 cells. The binding was inhibited by the addition of oligonucleotide DNA, although the intensity was less than plasmid DNA (Fig. 8). Taken together, these results suggest that MDP1 can bind A549 epithelial cells with its DNA-binding site.

Involvement of MDP1 and GAG Interaction in the Attachment/Invasion of Mycobacteria to A549 Cells—To elucidate the role of MDP1 in the attachment/invasion of mycobacteria, A549 epithelial cells were infected with BCG expressing GFP *in vitro*. CFUs determination showed around 7% of inoculated bacteria bound to cells after 6-h incubation. The interaction was then visualized by confocal laser microscopy. The result showed around 60% of bacteria bound to cells and 40% was invaded. Thus BCG not only bound to but also invaded into cells. This was consistent with previous reports (4, 32). In this condition, exogenous HA, heparin, DNA, and anti-MDP1 antibodies inhibited the interaction between GFP-expressing BCG and A549 cell (Fig. 9*A*). P31–50 suppressed BCG-A549 cell interaction as well (data not shown). Among these, the most potent inhibitor of the interaction was HA. Similar results were obtained from the experiment involving *M. tuberculosis* (Fig. 9*B*).

Inhibition of BCG Growth in Vivo by Treatment of Mice with HA—We have extended *in vitro* studies to *in vivo* animal experiments regarding the inhibitory role of GAGs in the interaction. Mice were injected intratracheally with either 1×10^6 CFU of BCG alone or BCG plus 50 μg of HA. Two weeks after the challenge, 40,875 ± 16,585 CFU was recovered from the lung of BCG-infected mice (Fig. 10). Treatment of such mice with HA resulted in a marked reduction of BCG growth (5,960 ± 4,530 CFU). This result was consistent with that of *in vitro* study, probably due to the prevention of mycobacterial attachment/invasion by exogenous HA.

DISCUSSION

The bacterial chromosomes are associated with abundant histone-like proteins that compactly hold the genome (33–35). It is generally accepted that such histone-like proteins participate functionally in the regulation of gene expression by altering three-dimensional genome structure (33–35). MDP1 is a presumed mycobacteria-specific histone-like protein, although it localizes on the cell wall, besides inside the cell (11, 15). In the present study we have focused on the physiological role of extracellularly occurring MDP1. We postulated that surface-exposed MDP1 acts as an adhesin in *Mycobacterium*-epithelial cell interaction based on the following facts. First, GAG is estimated as an infectious site of mycobacteria to epithelial cells (6). Second, DNA-binding protein tends to bind GAGs, as for example the heparin-Sepharose column is conventionally used to purify nuclear proteins. Third, MDP1 possesses six PAKK sequences that represent the heparin-binding site of HBHA (Fig. 2).

In the present study we explored interaction between MDP1 and GAGs and found that MDP1 directly bound to GAG at the DNA-binding site (Figs. 3–5). Affinity was determined by SPR analysis. Apparent rate constants were calculated by the basic model, because heparin and heparan sulfate are multivalent. There is a discrepancy at least in part by difference between the fitted model and real interaction mechanism and requires further investigation to obtain the actual kinetic constants for the interaction. MDP1 bound to A549 lung epithelial cells mainly through cell surface HA (Fig. 7). Anti-MDP1 antibody treatment inhibited the binding of mycobacteria, including BCG and *M. tuberculosis*, to A549 cells (Fig. 9). These findings indicate that MDP1 acts as an adhesin in the interaction with lung epithelial cells of the host. To our knowledge, this study demonstrates for the first time that bacterial pathogen utilizes extracellular DNA-binding proteins to attach host cells.

It has also been known that HBHA binds to GAGs and is identified as a mycobacterial adhesin (6). MDP1 and HBHA are structurally distinct proteins, although both proteins possess the conserved repetitive PAKK sequences in the C terminus (Fig. 2). Functionally, MDP1 binding to A549 cells was seen immediately after its addition, and >95% of A549 cells were MDP1-positive 60 min thereafter. In contrast, binding of HBHA was delayed, and >10% of cells were HBHA-negative

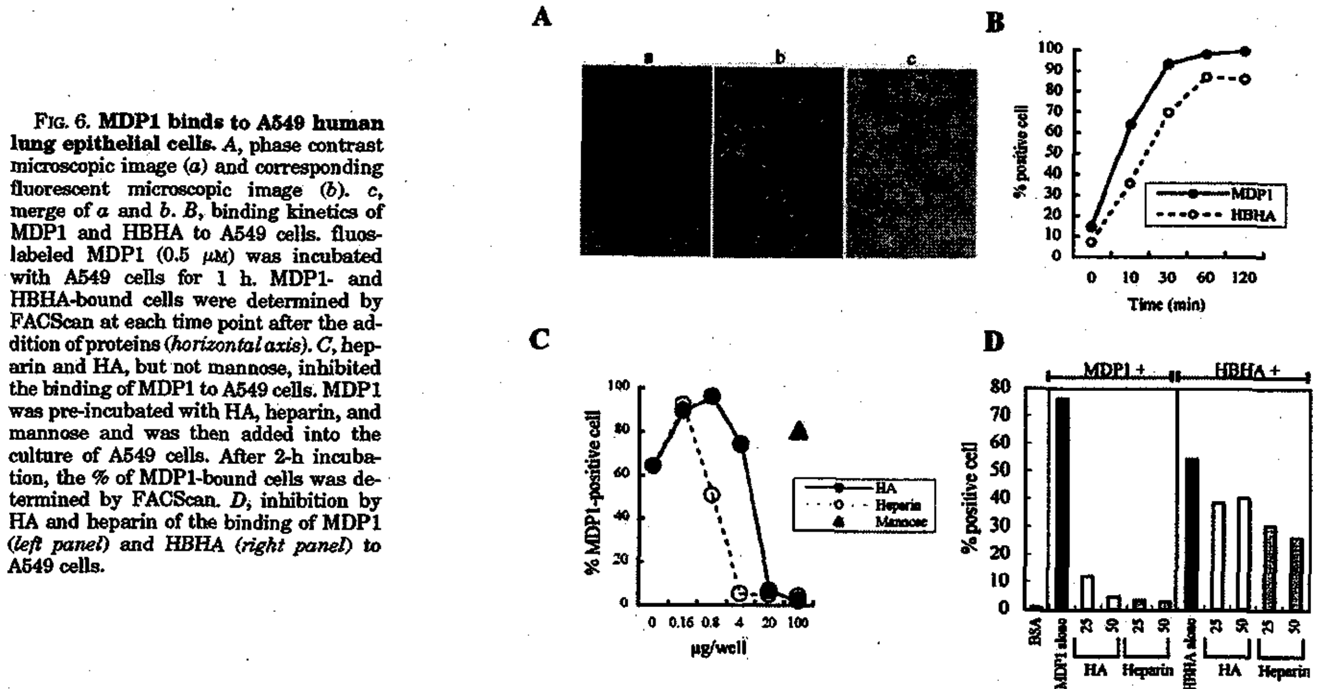

FIG. 6. **MDP1 binds to A549 human lung epithelial cells.** *A*, phase contrast microscopic image (*a*) and corresponding fluorescent microscopic image (*b*). *c*, merge of *a* and *b*. *B*, binding kinetics of MDP1 and HBHA to A549 cells. fluos-labeled MDP1 (0.5 μM) was incubated with A549 cells for 1 h. MDP1- and HBHA-bound cells were determined by FACScan at each time point after the addition of proteins (*horizontal axis*). *C*, heparin and HA, but not mannose, inhibited the binding of MDP1 to A549 cells. MDP1 was pre-incubated with HA, heparin, and mannose and was then added into the culture of A549 cells. After 2-h incubation, the % of MDP1-bound cells was determined by FACScan. *D*, inhibition by HA and heparin of the binding of MDP1 (*left panel*) and HBHA (*right panel*) to A549 cells.

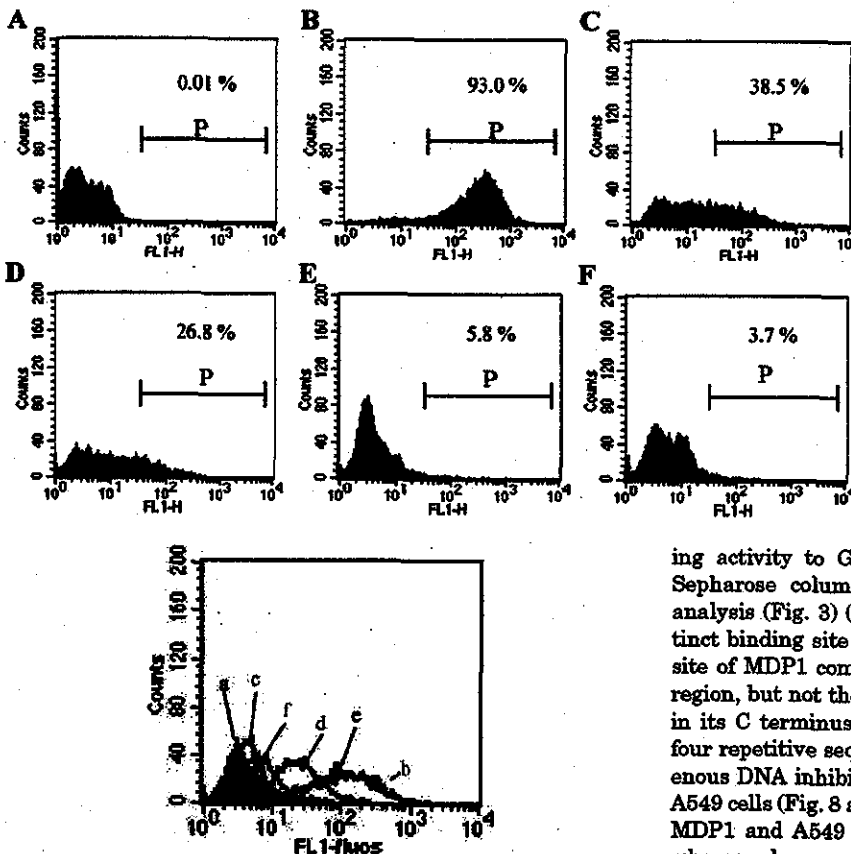

FIG. 7. **MDP1 binds to A549 cells through HA.** Untreated A549 cells bound to fluos-BSA served as negative controls (*A*) and to fluos-MDP1 as positive controls (*B*). MDP1 was added to the culture of cells pretreated with heparinase 1 (*C*), chondroitinase ABC (*D*), hyaluronidase (*E*), and combination of these three enzymes (*F*). Intensity of fluorescence was determined by FACScan 4–5 h after the culture.

FIG. 8. **DNA interferes in MDP1-A549 cell interaction.** A549 cells were incubated with fluos-BSA (*a*, *purple*), fluos-MDP1 alone (*b*, *green*), fluos-MDP1 plus plasmid DNA (pBSKS+) (*c*, *light blue*), synthetic poly(dG) (*d*, *pink*), rabbit control immunoglobulins (*e*, *dark blue*) (Sigma), rabbit anti-MDP1 polyclonal antibodies (*f*, *orange*) for 2 h. The binding was monitored by FACScan.

even at the plateau phase (Fig. 6*B*). The possible mechanism of the functional difference can be explained by the affinity experiment, which showed that MDP1 possessed stronger binding activity to GAGs than HBHA, as assessed by heparin Sepharose column chromatography and BIAcore biosensor analysis (Fig. 3) (26). Another possible mechanism is the distinct binding site of MDP1 and HBHA to GAGs. The binding site of MDP1 comprises aa 31–50, which is the DNA binding region, but not the region of six repetitive sequences of PAKK in its C terminus (Fig. 4), whereas that of HBHA comprises four repetitive sequences of PAKK (20, 21). As expected, exogenous DNA inhibited the binding of MDP1, but not HBHA, to A549 cells (Fig. 8 and data not shown). The interaction between MDP1 and A549 cells was through cell surface HA (Fig. 7), whereas heparan sulfate is presumed to be the site for HBHA (26).

Another major difference between MDP1 and HBHA is their localization. MDP1 retained mycobacterial cell walls, although HBHA is released to the extracellular milieu. The nature of MDP1 having strong affinity to both cell walls and GAG may be more favorable than HBHA to attach to host tissues. Recently Coutte *et al.* (36) found that the release of adhesin to the extracellular milieu is necessary for efficient colonization of *Bordetella pertussis*, which colonizes to the human respiratory

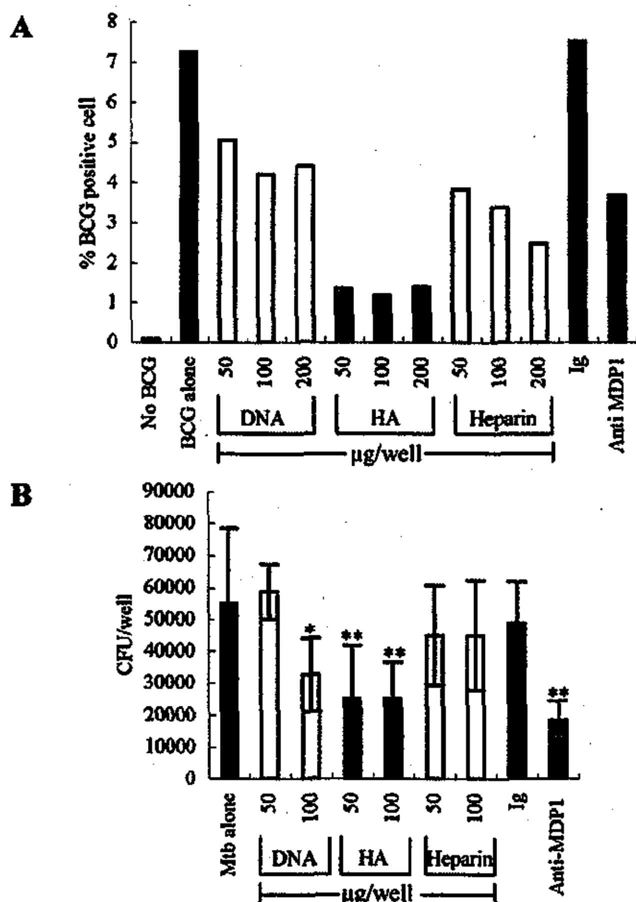

FIG. 9. **MDP1 is involved in the attachment/binding of mycobacteria to A549 cells.** *A*, the attachment/binding of BCG expressing GFP to A549 cells. BCG were pretreated with DNA, anti-MDP1 antibodies, HA, and heparin, and were then added into the culture of A549 cells. As controls for antibody experiments, rabbit control Ig (Sigma) was added. The binding was assessed by FACScan. The average of duplicated samples was presented. *B*, CFU of *M. tuberculosis* pretreated with GAGs and anti-MDP1 antibodies recovered from the coculture with A549 cells. Bacterial numbers were counted 3 weeks after the culture on the Middlebrook 7H11 agars. *Mtb*, *M. tuberculosis*. *, $p < 0.05$ and **, $p < 0.01$ as compared with Mtb alone by analysis of variance.

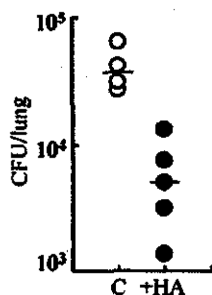

FIG. 10. **Inhibition of infection of BCG *in vivo* by treatment of mice with HA.** C57BL/6 mice were challenged intratracheally with either 1×10^6 CFU BCG alone (*C*) or BCG plus 50 μg of HA (*+HA*). All results are expressed as individual mouse data. *Bars* indicate averages. $p = 0.0143$ as compared between two groups by Mann-Whitney *U* test.

tract. HBHA induces bacterial aggregation, thereby mediating bacteria-bacteria interaction (6). These reports suggest that exported HBHA participates in the dispersal of bacteria from microcolonies for the spread of infection. This hypothesis explains the important finding by Pethe *et al.* (8): delay of dissemination of HBHA-lacking BCG and *M. tuberculosis* from the lung to spleen. Taking these considerations together, mycobacteria possess at least structurally and functionally two distinct adhesion proteins, such as extracellular MDP1 and HBHA.

It remains uncertain how MDP1 is expressed on cell walls of mycobacteria. An MDP1 gene does not encode signal sequence of secretion, and it does not contain the transmembrane domain of integration into the cell membrane. However, the large amounts of MDP1 that were localized on/in mycobacterial cell walls (Fig. 1) imply that some machinery is present that transports MDP1 outside the cell membrane rather than retention on the cell surface due to cell lysis of bacteria. Certain proteins that lack signal sequence and a transmembrane domain, such as ESAT-6 (37), CFP-10 (38), HSP65 and superoxide dismutase (SOD) (39, 40), and glutamine synthetase (39), are actively exported from mycobacteria and play significant roles in the pathogenesis. Further study is needed to clarify the issue of transportation of MDP1.

Hyaluronidase treatment of A549 cells abolished MDP1-A549 cell interaction (Fig. 7), and exogenous addition of HA reduced the binding of BCG and *M. tuberculosis* to A549 cells (Fig. 9), suggesting that cell surface HA is the key GAG for adherence of mycobacteria to lung epithelial cells. Mycobacterial adhesins other than MDP1 are likely to participate in binding to HA, because anti-MDP1 antibodies and DNA, inhibited the interaction between mycobacteria and A549 cells less than HA (Fig. 9*A*).

Based on the results of *in vitro* experiments, we have attempted the therapeutic intervention by HA using experimental infection with BCG in mice. Treatment of such mice with HA resulted in a marked reduction of BCG growth in the lung (Fig. 10). The results support the *in vitro* study that HA plays an important role in the interaction between mycobacteria and lung epithelial cells. This suggests that HA has potential for prophylactic interventions in mycobacterial infection.

HA has so far received little attention in the research of host-*Mycobacterium* interaction. HA is a polymer comprising repeating disaccharide units of (β1→4)D-glucuronate-(β1→3)*N*-acetyl-D-glucosamine (7). HA is the major component of the extracellular matrix and acts as a signaling molecule for cells depending on their size (41, 42). Professional phagocytes, such as macrophages, and professional antigen presenting cells, such as dendritic cells, play important roles in defense against mycobacterial infection in the lung (43). HA modulates the functions of dendritic cells and macrophages (42). Fragmented HA, which accumulates during inflammation, stimulates nitric oxide production (44), which is the major bactericidal effector against *M. tuberculosis* (43, 45). Furthermore, CD44, a major receptor of HA (46, 47), is recently identified as the site of mycobacterial entry to macrophages (48). We focused mainly on mycobacteria-epithelial cell interaction in this study, and our findings imply that HA plays an important role in interactions between mycobacteria and macrophages/dendritic cells. The precise mechanism of the interaction remains to be elucidated, and such study is currently underway in our laboratory.

Acknowledgments—We gratefully acknowledge Drs. Yukiko Nishiuchi, Shinji Maeda, and Clifton E. Barry III for helpful suggestions. We also acknowledge Mika Egami, Yasuyoshi Nishio, and Yukimi Kira in the technical assistances of fluorescein microscopy, flow cytometry, and BIAcore sensor analysis. In addition, we thank Megumi Matsumoto, Sara Matsumoto, and Takeshi Yamada for their heartfelt encouragement.

REFERENCES

1. Kagnoff, M. F., and Eckmann, L. (1997) *J. Clin. Invest.* **100,** 6–10
2. Bloom, B. R. (2002) *N. Engl. J. Med.* **346,** 1434–1435
3. Small, P. M., and Fujiwara, P. I. (2001) *N. Engl. J. Med.* **345,** 189–200
4. Bermudez, L. E., and Goodman, J. (1996) *Infect. Immun.* **64,** 1400–1406
5. Hernandez-Pando, R., Jeyanathan, M., Mengistu, G., Aguilar, D., Orozco, H., Harboe, M., Rook, G. A., and Bjune, G. (2000) *Lancet* **356,** 2133–2138

6. Menozzi, F. D., Rouse, J. H., Alavi, M., Laude-Sharp, M., Muller, J., Bischoff, R., Brennan, M. J., and Locht, C. (1996) *J. Exp. Med.* **184,** 993–1001
7. Hardingham, T. E., and Fosang, A. J. (1992) *FASEB J.* **6,** 861–870
8. Pethe, K., Alonso, S., Biet, F., Delogu, G., Brennan, M. J., Locht, C., and Menozzi, F. D. (2001) *Nature* **412,** 190–194
9. Arruda, S., Bomfim, G., Knights, R., Huima-Byron, T., and Riley, L. W. (1993) *Science* **261,** 1454–1457
10. Shimono, N., Morici, L., Casali, N., Cantrell, S., Sidders, B., Ehrt, S., and Riley, L. W. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15918–15923
11. Matsumoto, S., Yukitake, H., Furugen, M., Matsuo, T., Mineta, T., and Yamada, T. (1999) *Microbiol. Immunol.* **43,** 1027–1036
12. Furugen, M., Matsumoto, S., Matsuo, T., Matsumoto, M., and Yamada, T. (2001) *Microb. Pathog.* **30,** 129–138
13. Matsumoto, S., Furugen, M., Yukitake, H., and Yamada, T. (2000) *FEMS Microbiol. Lett.* **182,** 297–301
14. Lee, B. H., Murugasu-Oei, B., and Dick, T. (1998) *Mol. Gen. Genet.* **260,** 475–479
15. Shimoji, Y., Ng, V., Matsumura, K., Fischetti, V. A., and Rambukkana, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9857–9862
16. Brix, K., Summa, W., Lottspeich, F., and Herzog, V. (1998) *J. Clin. Invest.* **102,** 283–293
17. Muller, S., Scaffidi, P., Degryse, B., Bonaldi, T., Ronfani, L., Agresti, A., Beltrame, M., and Bianchi, M. E. (2001) *EMBO J.* **20,** 4337–4340
18. Yang, T., Witham, T. F., Villa, L., Erff, M., Attanucci, J., Watkins, S., Kondziolka, D., Okada, H., Pollack, I. F., and Chambers, W. H. (2002) *Cancer Res.* **62,** 2583–2591
19. Andersson, U., Wang, H., Palmblad, K., Aveberger, A. C., Bloom, O., Erlandsson-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H., and Tracey, K. J. (2000) *J. Exp. Med.* **192,** 565–570
20. Scaffidi, P., Misteli, T., and Bianchi, M. E. (2002) *Nature* **418,** 191–195
21. Naito, M., Ohara, N., Matsumoto, S., and Yamada, T. (1998) *Scand. J. Immunol.* **48,** 73–78
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
23. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H., and Hatfull, G. F., Snapper, S. B., Barletta, R. G., Jacobs, W. R., and Bloom, B. R. (1991) *Nature* **351,** 456–460
24. Molloy, A., Laochumroonvorapong, P., and Kaplan, G. (1994) *J. Exp. Med.* **180,** 1499–1509
25. Delogu, G., and Brennan, M. J. (1999) *J. Bacteriol.* **181,** 7464–7649
26. Pethe, K., Aumercier, M., Fort, E., Gatot, C., Locht, C., and Menozzi, F. D. (2000) *J. Biol. Chem.* **275,** 14273–14280
27. Naito, M., Ohara, N., Matsumoto, S., and Yamada, T. (1998) *J. Biol. Chem.* **273,** 2905–2909
28. Janicki, B. W., Chaparas, S. D., Daniel, T. M., Kubica, G. P., Wright, G. L., and Yee, G. S. (1971) *Am. Rev. Respir. Dis.* **104,** 602–604
29. Yang, V. C., Bernstein, H., Cooney, C. L., and Langer, R. (1987) *Appl. Biochem. Biotechnol.* **16,** 35–50
30. Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968) *J. Biol. Chem.* **243,** 1523–1535
31. Ohya, T., and Kaneko, Y. (1970) *Biochim. Biophys. Acta* **198,** 607–609
32. McDonough, K. A., and Kress, Y. (1995) *Infect. Immun.* **63,** 4802–4811
33. Drlica, K., and Rouviere-Yaniv, J. (1987) *Microbiol. Rev.* **51,** 301–319
34. Pettijohn, D. E. (1988) *J. Biol. Chem.* **263,** 12793–12796
35. Schmid, M. B. (1988) *Trends Biochem. Sci.* **13,** 131–135
36. Coutte, L., Alonso, S., Reveneau, N., Willery, E., Quatannens, B., Locht, C., and Jacob-Dubuisson, F. (2003) *J. Exp. Med.* **197,** 735–742
37. Sorensen, A. L., Nagai, S., Houen, G., Andersen, P., and Andersen, A. B. (1995) *Infect. Immun.* **63,** 1710–1717
38. Berthet, F. X., Rasmussen, P. B., Rosenkrands, I., Andersen, P., and Gicquel, B. (1998) *Microbiology* **144,** 3195–3203
39. Harth, G., and Horwitz, M. A. (1999) *J. Biol. Chem.* **274,** 4281–4292
40. Braunstein, M., Espinosa, B. J., Chan, J., Belisle, J. T., and Jacobs, W. R., Jr. (2003) *Mol. Microbiol.* **48,** 453–464
41. Camenisch, T. D., Spicer, A. P., Brehm-Gibson, T., Biesterfeldt, J., Augustine, M. L., Calabro, A., Jr., Kubalak, S., Klewer, S. E., and McDonald, J. A. (2000) *J. Clin. Invest.* **106,** 349–360
42. Termeer, C., Sleeman, J. P., and Simon, J. C. (2003) *Trends Immunol.* **24,** 112–114
43. Flynn, J. L., and Chan, J. (2001) *Annu. Rev. Immunol.* **19,** 93–129
44. McKee, C. M., Lowenstein, C. J., Horton, M. R., Wu, J., Bao, C., Chin, B. Y., Choi, A. M., and Noble, P. W. (1997) *J. Biol. Chem.* **272,** 8013–8018
45. Chan, J., Xing, Y., Magliozzo, R. S., and Bloom, B. R. (1992) *J. Exp. Med.* **175,** 1111–1122
46. Turley, E. A., Noble, P. W., and Bourguignon, L. Y. (2002) *J. Biol. Chem.* **277,** 4589–4592
47. Cichy, J., Pure, E., Turley, E. A., Noble, P. W., and Bourguignon, L. Y. (2003) *J. Cell Biol.* **161,** 839–843
48. Leemans, J. C., Florquin, S., Heikens, M., Pals, S. T., van der Neut, R., and Van Der Poll, T. (2003) *J. Clin. Invest.* **111,** 681–689

Jpn.J.Leprosy 73, 263-270(2004)

抗酸菌病原因子と宿主応答の分子機序

小林　和夫*
大阪市立大学大学院医学研究科感染防御学

〔受付：2004年7月6日〕

キーワード：血清診断、抗酸菌DNA結合蛋白質、宿主防御、細胞壁表層糖脂質、病変形成

抗酸菌感染症には結核、非結核性抗酸菌感染症やハンセン病などがあり、現在でも、多くの抗酸菌感染症患者が存在し、人類に甚大な健康被害を提供している。全世界では約20億人（全人口の1/3）が結核菌に既感染、毎年800万人が結核を発病、200万人が死亡している。今後10年間、少なくとも、8,000万人が発病、2,000万人が死亡することが推定されている。結核菌の病原性として、1）宿主防御機構からの逸脱や2）遅延型過敏反応（細胞性免疫応答の負の側面）の誘導があり、その結果、結核菌感染から発病に至る長期の潜伏期間、組織破壊を伴う肉芽腫炎症が特徴的である。結核菌病原因子として、細胞壁表層構成成分が関与していると考えられている。病原因子として、1）細胞壁表層糖脂質、2）lipoarabinomannan、3）補体活性化因子、4）熱ショック蛋白質や5）抗酸菌DNA結合蛋白質などがあり、これらの因子は抗酸菌や宿主に対する多機能分子である。抗酸菌感染症の制圧には抗酸菌病原因子や宿主応答の分子機序の解明、さらに、この機序の解明は新規治療戦略やワクチン開発に寄与するであろう。

はじめに

世界の年間総死亡は約5,400万人、その内訳として、循環器疾患（虚血性心疾患や脳血管障害など）：1,670万人、感染症：1,350万人、悪性新生物：700万人であり、感染症は現在でも全世界の総死亡の約1/4を占め、人類に大きな健康被害を招来している。感染症による死亡（1,350万人／年）の主要な原因として、急性呼吸器感染症（肺炎など）：396万人、後天性免疫不全症候群（AIDS、結核の合併を含む）：267万人、下痢性疾患：220万人、結核：200万人、マラリア：109万人や麻疹：89万人などがある（表1）。

抗酸菌感染症には結核、非結核性抗酸菌（nontuberculous mycobacteria：NTM）感染症やハンセン病などがあり、現在でも、多くの抗酸菌感染症患者が存在し、人類に甚大な健康被害を提供している。全世界では約20億人（全人口の1/3）が結核菌（*Mycobacterium tuberculosis*）に既感染、毎年800万人が結核を発病、200万人が死亡し、有病者は2,200万人である。今後10年間、少なくとも、8,000万人が発病、2,000万人が死亡することが推定されている。日本（2002年）では年間3.3万人（罹患率人口10万対：25.8）が結核を発病し、2.3千人（死亡率：1.8）が死亡し、有病者は3.2万人（有病率：25.4）、結核は単一病原体による感染症として、世界最大である。結核対策の課題として、1）急速な人口の高齢化に伴う高齢者結核の増加（70歳以上の占める割合：約40%）、

*Corresponding author：
〒545-8585　大阪府大阪市阿倍野区旭町1-4-3
TEL：06-6645-3745　FAX：06-6646-3662
E-mail：kobayak@med.osaka-cu.ac.jp
Web site：http://www.med.osaka-cu.ac.jp/hostdefense

2）地域格差の拡大、3）集団感染、4）結核菌の潜伏感染、5）多剤耐性結核菌の出現（初回耐性：1％、獲得耐性：20％、初回＋獲得耐性：2％）、6）有効な新規ワクチン開発、7）ヒト免疫不全ウイルス（HIV）感染症／後天性免疫不全症候群（AIDS）の重複感染などがある。

表1．世界における感染症による死亡数（2000）

感染症	死亡数:万人
全感染症	1,350
急性呼吸器感染症	396
AIDS（結核の合併を含む）	267
下痢性疾患	220
結核	200
マラリア	109
麻疹	89
参考：年間総死亡	5,400

世界保健機関、2000

結核菌の概要

結核菌（*M. tuberculosis*）の生物学的特徴として、1）細胞内寄生性、2）脂質成分に富む細胞壁、3）好気性、4）遅発育性、5）空気（飛沫核）感染、6）慢性炎症および7）遺伝子の解読などがある（表2）[1]。結核菌など抗酸菌は基本的に外毒素や内毒素非産生性であるが、例外的に*M. ulcerans*（西アフリカ諸国で猛威を奮っているBuruli潰瘍の原因菌）が外毒素（mycolactone、別名：polyketide toxin、宿主組織に壊死を惹起する）を産生する[2]。炎症病変や組織障害は結核菌に対する感染免疫応答過程で宿主から産生されるサイトカインをはじめとする生理活性物質に依存している。結核菌の細胞壁は長鎖脂肪酸（ミコール酸）に富み、グラム染色では難染色性を示す。そのため、抗酸性（Ziehl-Neelsen、Kinyoun）染色や蛍光染色が用いられる。抗酸性染色は石炭酸フクシンで加温染色後，塩酸アルコールで脱色，メチレンブルーで後染色する。抗酸菌は“赤い桿菌”として観察される。抗酸菌以外の通常細菌やヒト組織・細胞は後染色のメチレンブルーにより“青く”対比染色される。抗酸菌をオーラミンやローダミンなどの蛍光色素を用いる蛍光染色法も広く用いられている．分裂倍加時間は約12～15時間の遅発育菌であり、感染伝播は飛沫核（空気）感染による。宿主防御機構では、マクロファージ―サイトカイン―T細胞応答系、すなわち、細胞性免疫が役割を演じ、細胞内殺菌物質として、ガス状物質（反応性酸素化合物質や反応性窒素化合物質）が寄与している。その結果、結核菌感染者の約10％が一生涯において結核を発病する。病変は慢性炎症、肉芽腫、乾酪壊死、空洞形成や線維化などが特徴的である。*M. tuberculosis* H37Rvの全ゲノム塩基配列が解明された[3]。今後、遺伝子解析を基盤とした科学的戦略が推進され、分子／遺伝子標的を視点とした新規診断法、抗結核薬の開発、薬剤耐性獲得機構の解明や新規ワクチン開発が展開されるであろう[4]。

表2．結核菌の特徴

細胞内寄生性：	桿菌（0.2-0.6 x 1-10 μm）、宿主細胞、特に、マクロファージ内で抗菌機構から逃れて増殖
細胞壁：	脂質成分が豊富なため、疎水性であり、化学物質にも安定、グラム染色に難染色性、抗酸性
好気性：	酸素分圧の高い臓器（肺など）で増殖し、病変を形成
遅発育性：	至適温度：37℃、倍加時間：約12-15時間、培養集落形成に4-8週間
感染形式：	飛沫核／空気感染
病原性：	慢性炎症、肉芽腫、乾酪壊死、空洞形成、線維化
遺伝子：	全ゲノム（約4.41 Mb）の解読

結核菌の病原性

結核菌の病原性（表3）として、1）宿主防御機構からの逸脱や2）遅延型過敏反応（細胞性免疫応答の負の側面）の誘導があり、その結果、結核菌感染から発病に至る長期の潜伏期間、組織破壊を伴う肉芽腫炎症が特徴的である[5]。

表3．抗酸菌の病原性と病原因子

病原性	宿主防御機構から逸脱 遅延型過敏反応の誘導	
	化学的性状	生物学的活性
病原因子	細胞壁表層ミコール糖脂質（TDM、SL）	肉芽腫誘導やP-L融合の阻害
	Lipoarabinomannan	マクロファージ活性化の阻害、TNF-α誘導による組織障害、Th1 細胞応答抑制性サイトカインの誘導
	補体活性化因子	補体受容体3や4、オプソニン化による貪食能の亢進
	65 kDa 熱ショック蛋白	宿主の免疫的交叉反応性による自己免疫の誘導
	DNA結合蛋白	接着や侵入の促進
	外毒素	mycolactone（*M. ulcerans*）

結核菌の潜伏感染

肺結核患者（特に、喀痰塗抹陽性）から曝露された約30％に結核菌感染が成立し、感染者の約10％が一生涯において結核を発病する。有効な感染防御応答により、90％の感染者は結核菌を封じ込め、発病を回避している。しかし、結核菌は宿主

内で潜伏感染している。潜伏感染した結核菌は宿主免疫機構の破綻（老化、免疫抑制薬／副腎皮質ステロイド薬投与、栄養障害、HIV感染／AIDSなど）により、発育・増殖を再開し、結核を発病するに至る（内因性再燃）。人類の約1/3が結核菌に潜伏感染している事実を考慮すると、潜伏感染機序を解明することは新規抗結核薬やワクチン開発を促進し、その結果、結核制圧に寄与するであろう[6]。潜伏感染した結核菌の特性として、1）定常期に発現するσ因子（*sigF*遺伝子、対数増殖期には未発現）や2）糖代謝から脂質代謝への変換が知られている。*sigF*欠損結核菌は肉芽腫内で生存不能であり、肉芽腫内生存結核菌はglyoxylate shunt（脂肪酸から糖代謝への変換酵素系、哺乳動物の冬眠におけるエネルギー代謝変換に類似）を亢進させている[7]。

肉芽腫炎症と感染防御の統御

結核菌など抗酸菌は細胞内寄生病原体であり、宿主防御にマクローファージ—サイトカイン—CD4陽性1型ヘルパーT（Th1）細胞応答系、細胞性免疫が貢献している。細胞性免疫の起動サイトカインとして、interleukin（IL）-12、IL-18やinterferon（IFN）-γがTh1細胞分化や活性化など、重要な役割を演じている[8]。しかし、結核菌感染に対する遅延型過敏反応を含む細胞性免疫の発現は抗結核菌防御と組織傷害に貢献、すなわち、功罪の二面性（諸刃の剣）を表現する[9]。また、遺伝的因子として、ヒト第2染色体に存在する遺伝子（*NRAMP1*：natural resistance associated macrophage protein 1、別名＝*SLC11A1*）が感染防御に関与し、この機能はマクロファ-ジに表現されている。初回の結核菌曝露の場合、宿主の炎症応答は非特異的であり、普遍的な細菌感染に対する炎症応答に類似している。感染約4～6週後に乾酪壊死を伴う肉芽腫炎症が生じ、また、結核菌ツベルクリン蛋白質抗原に遅延型過敏反応、ツベルクリン皮内反応が成立する（図1）。この機序として、病変部において炎症惹起性サイトカイン（IL-1やtumor necrosis factor α：TNF-α）、Th1細胞関連サイトカイン（IL-12、IL-18、IFN-γ）や単球走化性ケモカイン（monocyte chemoattractant protein 1：MCP-1やmacrophage inflammatory protein 1αなど）が産生され、マクロファ-ジの局所的集積（肉芽腫）、加えて、細胞性免疫（遅延型過敏反応を含む）が誘導される。

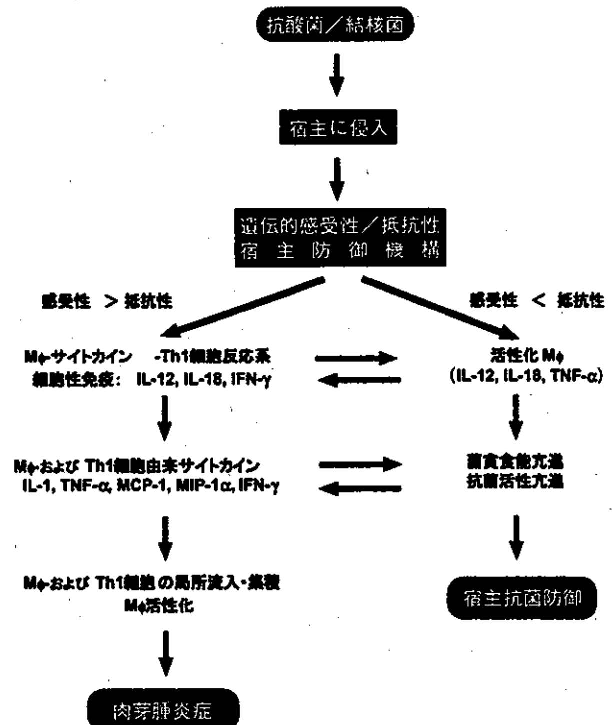

図1．抗酸菌／結核菌感染における宿主細胞および機能分子応答機構
Mo：マクロファージ

結核菌の病原因子

結核菌病原因子として、細胞壁表層構成成分が関与していると考えられている[1]。病原因子として、1）細胞壁表層糖脂質、2）lipoarabinomannan、3）補体活性化因子、4）熱ショック蛋白質や5）抗酸菌DNA結合蛋白質などがある（表3）。本稿では、特に、細胞壁表層糖脂質や抗酸菌DNA結合蛋白質について、概説する。

細胞壁糖脂質

結核菌の脂質は乾燥菌体重量の10%以上、細胞壁の20%以上を構成し、他の一般細菌に比し、極めて多い。事実、結核菌の全ゲノムは約4.4 Mb

（大腸菌：4.6 Mb）であり、蛋白質を規定している遺伝子は約4000、脂肪酸代謝に関与している酵素は250以上、大腸菌が50であることから、結核菌の脂質代謝が極めて旺盛であることが遺伝子情報からも判明している[3]。結核菌細胞壁の脂質として、mycolyl-arabinogalactan-peptidoglycan complex、lipoarabinomannan（LAM）、lipomannan、phosphatidyl-*myo*-inositol、sulfolipid（SL）、trehalose 6,6'-dimycolate（TDM）/cord factor、phenolic glycolipidやlipooligosaccharidesなどの糖脂質が特徴的である[10]。

特に，アシル化trehalose脂質化合物であるTDM/cord factorやSL（図2）が結核菌に特徴的であり、結核菌-宿主関係、すなわち、病原性や毒性の発現に関与している。また、“抗酸性”に主として関与する菌体表層成分はミコール酸などの脂質成分であり、ミコール酸は天然で稀なα位に分枝鎖、β位に水酸基を持つ長鎖脂肪酸（結核菌では炭素数：60～90）である。TDMの構造—活性連関で側鎖炭素鎖長が病原性に関与している[11]。

宿主マクロファージは結核菌を貪食し、食胞体（phagosome）を形成するが、アシル化trehalose脂質化合物（SLやTDM）が加水分解酵素を含むリソゾーム（lysosome）との融合（P-L fusion）を阻害することにより、酸性化されず、食胞体内、すなわち、宿主細胞内での生存を可能にしている。

肉芽腫炎症は発症機序により、異物性（T細胞非依存性）および過敏性（T細胞依存性）に大別（図3）される。TDMを無胸腺ヌードマウスや未免疫マウスに投与することにより、肉芽腫を惹起できること、TDM免疫マウスではTDM誘導肉芽腫が増強されることや遅延型過敏反応／細胞性免疫の指標である足蹠腫脹反応が誘導されることから、TDMは異物性および過敏性の両機序を介して、肉芽腫を形成する[12]。事実、TDM誘導肉芽腫病変は多量の細胞性免疫起動性サイトカイン（IL-12やIFN-γ）を含み、活動性に伴い、消長する[12]。従って、結核肉芽腫の発現に異物性および過敏性の両機序が複合関与し、結核肉芽腫は混合性肉芽腫である。肉芽腫形成には病変局所への血中単球の流入や活性化が必須であり、このため、局所のcc-ケモカイン産生や血管新生が重要な役割を演じる。TDMは血管内皮細胞細胞増殖因子（vascular endothelial growth factor：VEGF）を誘導し、局所の血管新生に寄与している[13]。加えて、TDMは宿主免疫担当細胞（胸腺や脾臓）にアポトーシスを誘導し，その結果，細胞内寄生病原体の増殖や生存を困難にし，さらに胸腺内自己反応性T細胞を除去，Th1/Th2細胞の分化を制御することにより自己免疫疾患の発症を防止している可能性がある[14]。しかし、SLはP-L fusion阻害（宿主細胞内生存）を発揮したが、炎症・免疫惹起やアポトーシス誘導活性を全く示さず、TDMと対照的である[12]～[14]。アシル化trehalose脂質化合物は結核菌細胞壁表層に存在し、1）結

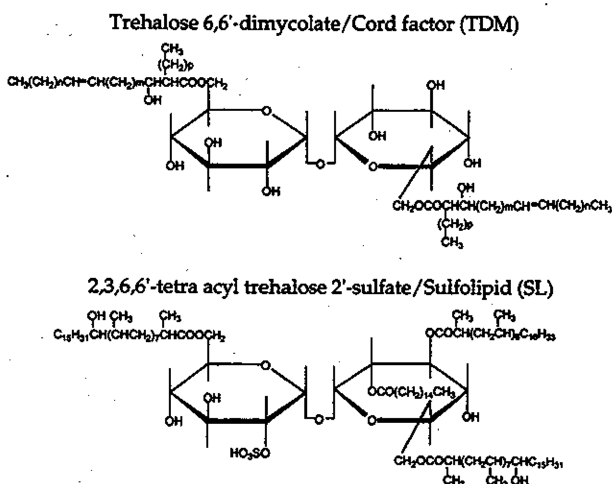

図2．アシル化トレハロース脂質の化学構造

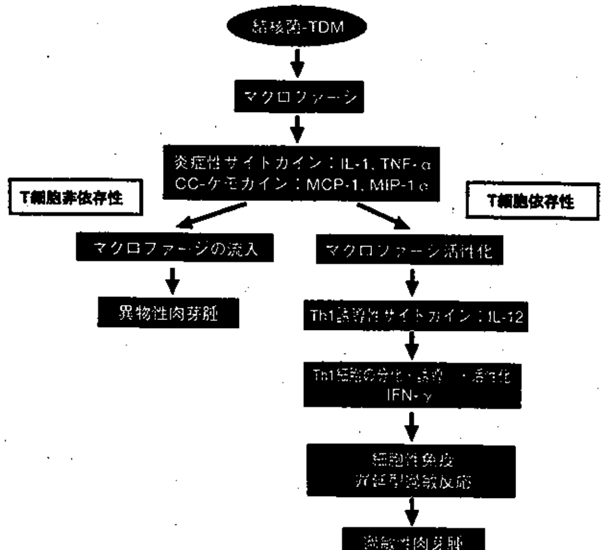

図3．結核性肉芽腫炎症機序と細胞壁TDM

核菌の宿主細胞内生存、2）炎症・免疫惹起物質（肉芽腫炎症、遅延型過敏反応、血管新生など）や3）アポトーシス誘導活性を有する多機能分子である（図4）。

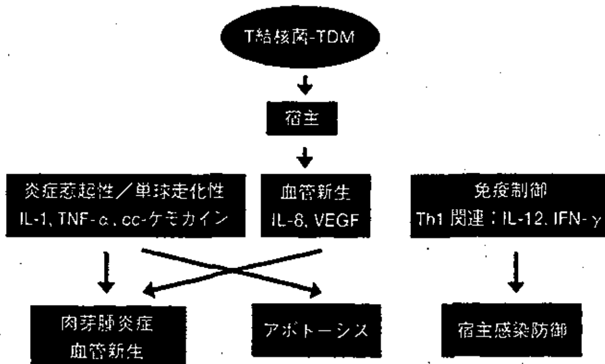

図4．結核菌細胞壁TDMと宿主応答の分子機序

抗酸菌DNA結合蛋白質 (mycobacterial DNA-binding protein 1：MDP1)

遅発育型抗酸菌が定常期や休眠期において最も大量に発現する蛋白質であるMDP1（分子量：28 kDa）は抗酸菌体内や表層に存在する抗酸菌特異的蛋白質である。機能的に細胞質内MDP1は核酸結合性を示し、転写および翻訳阻害活性を有し、そのため、休眠機構、さらに、潜伏感染に関与していることが示唆されている。他方、菌表層MDP1の生物学的意義は不明であった。MDP1が宿主細胞表面に存在するglycosaminoglycan、特に、ヒアルロン酸に結合し、抗酸菌の接着／侵入に関与することが判明した[15]。ヒアルロン酸は細胞外マトリックスの主要構成成分として組織の形態決定や細胞浸潤に関与、さらに、細胞表面受容体を介して細胞を活性化・増殖や分化を促す生理活性物質でもある。低分子ヒアルロン酸はCD44介在性にマクロファージを集積させ、抗酸菌殺傷活性を有する一酸化窒素産生を促す。加えて、ヒアルロン酸は抗原提示樹状細胞の活性化を誘導する。すなわち、ヒアルロン酸は宿主感染防御の成立に関与している。ヒアルロン酸は多機能分子であり、ヒアルロン酸が抗酸菌に対する宿主細胞受容体としての機能以外に、結核病態形成や防御への関与を示唆している。

病原因子の将来展望・臨床応用

細胞壁表層糖脂質や抗酸菌DNA結合蛋白質に関し、臨床医学的視点（診断、治療および予防）に立脚し、現在の状況を概説する。

抗酸菌細胞壁糖脂質を抗原とした血清診断

感染宿主は抗酸菌細胞壁糖脂質に対し抗体産生など液性免疫応答を表現するため、抗酸菌細胞壁糖脂質抗原を用いた血清診断が開発されている。結核菌細胞壁糖脂質抗原（TDM）による血清診断は感度（80%）および特異度（95%）であり、特に、喀痰塗抹陰性や培養陰性結核に有用な診断方法であることが示唆されている[16]。

M. avium complex（MAC）感染症は結核など抗酸菌感染症の約20%を占める。MACは環境菌であり、普遍的に存在し、臨床および病原体診断の確定には臨床経過を考慮するため、長期間を要する。MAC特異的細胞壁表層糖ペプチド脂質（GPL）抗原（図5）を用いた迅速・簡便血清診断法は感度および特異度ともに良好な成績（90%以上）を示し、また、血清抗GPL抗体価はMAC感染症の疾患活動性を反映した[17]。従って、GPL抗原を用いた血清診断はMAC感染症の診断や疾患活動性の評価に有用であり、今後、大規模臨床試験など、臨床応用が期待される。

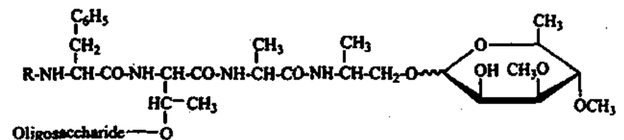

図5．MAC特異的細胞壁表層糖ペプチド脂質（GPL）の化学構造

抗酸菌の宿主細胞接着や侵入を標的とした治療・予防介入

抗酸菌の宿主細胞接着や侵入に関するMDP1-ヒアルロン酸関係をマウス気道抗酸菌感染モデルにて検証した。その結果、ヒアルロン酸をBCGと同時、或いは、感染成立後に投与することにより、

感染菌数が著しく減少した。BCG-宿主細胞結合をヒアルロン酸が阻害し、定着菌数が減少した結果であろう[15]。現行の治療戦略に抗酸菌の接着や侵入を標的とした治療予防戦略は存在しないため、MDP1やヒアルロン酸が結核予防や治療戦略の開発に有望な候補になる可能性を示している。

おわりに

抗酸菌病原因子や宿主応答の分子機序は未解明な部分が多く、抗酸菌感染症の制圧には抗酸菌病原因子や宿主応答の分子機序の解明、さらに、この機序の解明は新規治療戦略やワクチン開発に寄与するであろう。

謝 辞

本稿に示した著者の成績は主として教室員、大学院生や共同研究者の成果であり、感謝に堪えない。本研究は厚生労働省　厚生労働科学研究費補助金（新興・再興感染症研究事業）、文部科学省科学研究費補助金（特定領域研究および基盤研究C）、日米医学協力研究会結核・ハンセン病専門部会および大阪市立大学都市問題研究により支援された。

文 献

1）Smith I: *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 16: 463-496, 2003.

2）George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, Small PL: Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science 283: 854-857, 1999.

3）Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE, Tekaia III F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream M-A, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston J E, Taylor K, Whitehead S, Barrel BG: Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393: 537-544, 1998.

4）Young DB: Building a better tuberculosis vaccine. Nat Med 9: 503-504, 2003.

5）Hingley-Wilson SM, Sambandamurthy VK, Jacobs WR: Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. Nat Immunol 4: 949-955, 2003.

6）Bloom BR: Tuberculosis. The global view. N Engl J Med 346: 1434-1435, 2002.

7）McKinney JD, Honer zu Bentrup K, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, Swenson D, Sacchettini JC, Jacobs WR Jr, Russell DG: Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406: 735-738, 2000.

8）North RJ, Jung Y J: Immunity to tuberculosis. Annu Rev Immunol 22: 599-623, 2004

9）Kobayashi K, Kaneda K, Kasama T: Immunopathogenesis of delayed-type hypersensitivity. Microsc Res Tech 53: 241-245, 2001.

10）Brennan PJ, Nikaido H: The envelope of mycobacteria. Annu Rev Biochem 64: 29-63, 1995.

11）Ueda S, Fujiwara N, Naka T, Sakaguchi I, Ozeki Y, Yano I, Kasama T, Kobayashi K: Structure-activity relationship of mycoloyl glycolipids derived from *Rhodococcus* sp. 4306. Microb Pathog 30: 91-99, 2001.

12）Yamagami H, Matsumoto T, Fujiwara N, Arakawa T, Kaneda K, Yano I, Kobayashi K: Trehalose 6,6'-dimycolate (Cord factor) of *Mycobacterium tuberculosis* induces foreign-body- and hypersensitivity-type granulomas in mice. Infect Immun 69: 810-815, 2001.

13）Saita N, Fujiwara N, Yano I, Soejima K,

Kobayashi K: Trehalose 6,6'-dimycolate (Cord factor) of *Mycobacterium tuberculosis* induces corneal angiogenesis in rats. Infect Immun 68: 5991-5997, 2000.

14) Hamasaki N, Isowa KI, Kamada K, Terano Y, Matsumoto T, Arakawa T, Kobayashi K, Yano I: In vivo administration of mycobacterial cord factor (trehalose 6,6'-dimycolate) can induce lung and liver granulomas and thymic atrophy in rabbits. Infect Immun 68: 3704-3709, 2000.

15) Aoki K, Matsumoto S, Hirayama Y, Wada T, Ozeki Y, Niki M, Domenech P, Umemori K, Yamamoto S, Mineda A, Matsumoto M, Kobayashi K: Extracellular mycobacterial DNA-binding protein 1 participates in *Mycobacterium*-lung epithelial cell interaction through hyaluronic acid. J Biol Chem 2004 (in press).

16) Maekura R, Kohno H, Hirotani A, Okuda Y, Ito M, Ogura T, Yano I: Prospective clinical evaluation of the serologic tuberculous glycolipid test in combination with the nucleic acid amplification test. J Clin Microbiol 41: 1322-1325, 2003.

17) Kitada S, Maekura R, Toyoshima N, Fujiwara N, Yano I, Ogura T, Ito M, Kobayashi K: Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. Clin Infect Dis 35: 1328-1335, 2002.

Molecular Pathogenesis of Mycobacterial Diseases

Kazuo Kobayashi*
Department of Host Defense, Osaka City University Graduate School of Medicine Osaka, Japan

[Received: 6 July, 2004]

Key words : disease progression, host defense, mycobacterial DNA binding protein, serodiagnosis, surface glycolipid

Mycobacterial diseases, including tuberculosis, leprosy, and disease due to nontuberculous mycobacteria, are the major cause of death from infectious diseases around the world. About one-third of the world population is latently infected with *Mycobacterium tuberculosis*. Over 8 million new cases and nearly 2 million deaths occur each year. Tuberculosis presents a significant health threat to the world. The pathogenicity of mycobacteria is related to their ability to escape killing by ingested macrophages, latent infection, and induce delayed type hypersensitivity. This has been attributed to several components of the mycobacterial cell wall, such as surface glycolipids, lipoarabinomannan, complement activation factor, heat-shock protein, and mycobacterial DNA binding protein. From the aspect of my research interests, I have focused on mycobacterial glycolipids and mycobacterial DNA binding protein in this article. Surface molecules of mycobacteria exert pleiotropic activities in both the microbe and host, and thus participate in the pathogenesis of mycobacterial diseases. The better understanding of mycobacterial pathogenicity may open the new avenue for the development of therapeutic and prophylactic interventions.

*Corresponding author :
Department of Host Defense, Osaka City University
Graduate School of Medicine Osaka, Japan
TEL:06-6645-3745 FAX:06-6646-3662
E-mail:kobayak@med.osaka-cu.ac.jp
Web site:http://www.med.osaka-cu.ac.jp/hostdefense

感染症と移植
PART 4

結　核
—世界最大の感染症—

小 林 和 夫*

Tuberculosis : Tuberculosis as a major public health threat
世界では，約20億人(人類の1/3)が結核菌(*Mycobacterium tuberculosis*)に既感染，毎年800万人が結核を発病，200万人が死亡している．日本では，年間3万3,000人(罹患率人口10万対：25.8)が結核を発病，2,300人(死亡率：1.8)が死亡し，結核は単一病原体による世界最大の感染症である．結核制圧目標は，喀痰塗抹陽性肺結核罹患率：0.1/10万人以下であるが，日本の現状は9.4であり，制圧目標には程遠い．類縁である非結核性抗酸菌(*M. avium* complex や *M. kansasii* など)感染症は結核など抗酸菌感染症の約20%を占める．非結核性抗酸菌は環境菌であり，また，*M. avium* complex は抗微生物薬に対し多剤耐性を示すため，治療に難渋している．結核対策の重点項目として，① 直接監視下短期抗結核化学療法の普及，② 潜在性結核菌感染対策，③ HIV感染/AIDSと結核の重複感染に対する効果的な戦略，④ 多剤耐性結核に対する抗結核薬，⑤ 迅速，簡便な診断法，⑥ 有効なワクチンの開発，⑦ 集団や院内感染対策，が推進され，結核が制圧されることを期待している．

*Kazuo Kobayashi**

key words : 再興感染症(re-emerging infectious disease)，多剤耐性結核(multidrug-resistant tuberculosis)，直接監視下短期抗結核化学療法(directly observed treatment, short course : DOTS)，施設内感染(intrainstitutional infection)，潜在性結核感染(latent tuberculosis infection)

結核は代表的な再興感染症であり，世界および日本においても，単一病原体感染症として，人類に甚大な健康被害を提供している．世界の感染症による年間死亡(2000年)は1,350万人(総死亡：5,400万人の約1/4)を占め，感染症死亡の内訳では，呼吸器感染症：約400万人，後天性免疫不全症候群(AIDS)(結核合併を含む)：約270万人，下痢性疾患：220万人，結核：200万人，マラリア：110万人である[1,2](**表1**)．

呼吸器感染症や下痢性疾患の原因病原体は単一でなく，多種多様である．そのため，世界保健機関やG8サミット[3]は，① ヒト免疫不全ウイルス(HIV)感染/AIDS，② 結核，③ マラリア(熱帯熱マラリア)による死亡が年間約500万人，患者発生が3億人であることから，これら三大疾患を最重要感染症に認定し，世界が協調して対策を構築することを宣言している．また，1993年に世界保健機関，1999年に厚生省(現厚生労働省)が“結核緊急事態宣言”を発表，結核問題を再認識し，制圧対策を推進している．

結核の発生動向

世界では，約20億人(全人口の1/3)が結核菌(*Mycobacterium tuberculosis*)に既感染(ほとんどは

*Department of Host Defense, Osaka City University Graduate School of Medicine 大阪市立大学大学院医学研究科感染防御学

表1　世界における感染症による死亡数(2000年)

感染症	死亡数(万人)
全感染症	1,350
急性呼吸器感染症	396
AIDS(結核合併を含む)	267
下痢性疾患	220
結核	200
マラリア	110
麻疹	90
破傷風	38
百日咳	30
性感染症	18
髄膜炎	17

(世界保健機関，2000)

表2　世界および日本の結核発生動向

	結核菌既感染者数	年間死亡数	新規登録患者数	有病者数
世界	20億人	200万人	800万人	2,200万人
日本	0.3億人	0.23万人	3.3万人	3.2万人

潜在性)，毎年800万人が結核を発病，200万人が死亡し，有病者は2,200万人である[4]．なお，結核関連死亡(結核，結核とHIV感染/AIDSの重複)は350万人である．すなわち，毎秒，新規結核患者が発生し，毎15秒に1人が結核で死亡，1人の無治療結核患者が年間10～15人の感染者を生じさせ，世界の人口の約1%が毎年，結核菌に感染している．なお，結核菌感染後の生涯にわたる発病率は5～10%である．世界保健機関は，今後約20年間に10億人の新規感染者が発生，1億5,000万人が結核を発病，そして，3,600万人の結核死亡を予測している[5]．

日本では，年間3万3,000人(罹患率人口10万対：25.8)が結核を発病，2,300人(死亡率：1.8)が死亡し，有病者は3万2,000人(有病率：25.4)であり，結核は単一病原体による最大の感染症である[6](**表2**)．

日本における結核対策の課題(**表3**)として，① 急速な人口の高齢化に伴う高齢者結核の増加(70歳以上の占める割合：約42%)，② 国内地域格差の拡大(最高罹患率は大阪市：74.4，最低は長野県：12.5)，③ 集団や院内感染の続発および増加(1998～2001年合計：166件，結核集団感染の定義は同一の感染源が，2家族以上にまたがり，20人以上に結核を感染させた場合，なお，発病者1人は感染者6人と換算する)，④ 多剤耐性結核菌の出現(初回耐性：1～2%，再治療：10～20%，多剤耐性結核菌の定義はイソニアジド：INHとリファンピシン：RIFに少なくとも同時耐性)，⑤ 特異的，迅速かつ簡便な結核菌感染の検査法の開発，⑥ 潜在性結核菌感染対策などがある．加えて，HIV感染/AIDSが着実に増加している現状[7]を考慮した場合，日本においても，⑦ HIV感染/AIDSと結核の重複感染は将来的に重要な課題となることが想定される．今後，再興感染症として，結核の重要性を認識し，確実な治療や予防対策を推進することが肝要である．

表3　結核対策の課題

- 高齢者結核の増加
- 国内地域格差の拡大
- 集団や院内感染の続発および増加
- 多剤耐性結核菌の出現
- 特異的，迅速かつ簡便な結核菌感染の検査法の開発
- 潜在性結核菌感染対策
- HIV感染/AIDSと結核の重複感染

感染症の発生動向将来予測として，社会基盤の整備，抗微生物化学療法やワクチンで治療・予防可能な疾患(下痢性疾患，肺炎，麻疹など)は減少することが考えられるが，HIV感染/AIDSや結核は，今後も現状維持あるいは増加することが予測されている[8]．

結核の増加要因

結核の増加要因は，社会要因，宿主要因，および病原体要因に大別される[9]．

社会要因として，国際化，交通機関の発達による高速・大量移動，都市化による過密，貧困，受診や診断の遅延，結核対策の軽視などが寄与している．宿主要因として，感染抵抗力の減弱(人口の高齢化，糖尿病，慢性腎不全，HIV感染/AIDS，免疫抑制薬/臓器移植や免疫疾患など)が

表4 結核の増加要因

社会要因
- 高速，海外旅行および移民の増加(年間海外旅行者：約1,600万人，海外からの旅行者：約500万人，外国人登録者：約150万人)
- 貧困や衛生状態の低下を伴う人口過密都市
- 受診や診断の遅延
- 結核対策の不備や軽視

宿主要因
- 易感染性宿主の増加(人口の高齢化，糖尿病，慢性腎不全，HIV感染/AIDS，免疫抑制薬/臓器移植や免疫疾患など)

病原体要因
- 薬剤耐性結核菌の出現
- 病原性の変化

表5 薬剤耐性結核の出現状況 (%)

	いずれの1薬剤	多剤耐性
全体	12.6	2.2
初回耐性	9.9～10.7	1.0～1.4
獲得(再治療)耐性	23.3～36.0	9.3～13.0

いずれの1薬剤：INH，RIF，エサンブトール(EMB)，ストレプトマイシン(SM)

易感染性を招来している．また，病原体要因として，薬剤耐性結核菌の出現および病原性の変化などが増加に関与している(**表4**)．

特に，憂慮すべき増加要因は，① HIV感染/AIDS，および，② 多剤耐性結核(MDR-TB)の出現である．

2003年12月現在，世界のHIV感染者/AIDS患者は4,200万人，結核菌とHIVの重複感染は約1,400万人，結核を発症した患者でHIV陽性は約8%を占めている[10]．結核菌感染に対する宿主防御は細胞性免疫(マクロファージ—サイトカイン—1型ヘルパーTリンパ球連関)の発現に依存している[11～13]が，HIV感染/AIDSは細胞性免疫を破壊するため，結核菌感染や発病を惹起しやすくする．実際，HIV陽性者の発病の相対危険度はHIV陰性者の約10倍である[14]．また，AIDS死亡の約10%が結核を直接原因としている[5]．HIV感染/AIDS蔓延の防止は，感染経路(経血液，性的接触，母子感染)の遮断であり，性的接触，特に不特定多数との性交渉の回避，コンドーム使用による安全な性行為が重要である[10]．

薬剤耐性結核の原因は，不適切な結核医療，すなわち，抗結核化学療法薬の不適切な選択や使用，治療中断や脱落であり，医療関係者や患者の対応に起因するman-made diseaseである[15,16]．全世界で5,000万人以上が多剤耐性結核菌に既感染し，医療費は薬剤感受性結核に比し3～100倍を要し，さらに，再発率(28%)がきわめて高く，結核制圧対策に大きな課題を提供している(**表5**)．

薬剤耐性，特に，多剤耐性結核に有効な抗結核薬や制圧戦略の開発は急務の課題である[17]．薬剤耐性結核の出現を防止する効果的な戦略は，薬剤感受性結核を確実に治療，そして，治癒させることであり，世界保健機関は直接監視下短期抗結核化学療法(directly observed treatment, short course：DOTS)を推奨している．DOTSの基本は，標準的な抗結核化学療法薬として，INH，RIF，EMBおよびピラジナミドの4薬を併用し，かつ，患者の服薬を毎日確認することである(面前服用も含む)．1薬剤当たりの耐性菌出現頻度は$1/10^{6\text{-}8}$であるため，多薬剤を併用することにより，耐性結核菌の出現頻度を低下させることが可能である[4,5]．

なお，米国疾病管理予防センター，米国感染症学会や米国胸部学会の勧告では，結核菌のSM耐性が増加しているため，SMの選択順位は低下している[18]．

集団感染や施設内(院内)感染対策

日本における集団感染は166件(1998～2001年の合計)であり，事例は増加傾向を示している[19]．主要な発生場所は，学校(38%)，事業所(29%)，病院(20%)であるが，その他(施設，遊興場，飲食店など)にも拡大している．

この理由として，① 社会的活動の旺盛な世代における結核菌未感染者の増加→初感染結核や発病の増加(結核の場合，外来性再感染が比較的少ないため)，② 国民や医療従事者の結核に対する関心の低下により，受診および診断の遅延→