comparatively low rate and the small size of the clusters suggest that the high resistance of *M. tuberculosis* in Heilongjiang Province is not related to recent transmission but, rather, may be related to reactivation or inappropriate therapy. However, the clustering rate is still increasing and was much higher in late 2008 (12.8%) than in 2007 (6.4%), suggesting that more effective control strategies are needed.

This is the first report describing the molecular epidemiology of *M. tuberculosis* isolated from patients with pulmonary TB in Heilongjiang Province, China. The low clustering rate in our area indicates that only mild active transmission occurred in the time period studied. We defined the most suitable MIRU-VNTR locus set for analyzing the *M. tuberculosis* isolates in Heilongjiang Province, where Beijing family strains are prevalent. In our hands, the 15-locus set provided a high degree of discrimination; the 10-locus set was shown to be ideal for use in first-line molecular typing in future research although we still need to examine the discriminatory power of the rest of the recommended loci.

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Apoptosis-Inducing Activity of Clofazimine in Macrophages[▽]

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Clofazimine is a riminophenazine compound which has been used for the treatment of leprosy since the 1960s. Although the drug is effective in the management of leprosy reactions because of its anti-inflammatory activity, the mechanism leading to the cessation of inflammation is not well understood. In the present study, it was shown that clofazimine exhibits apoptosis-inducing activity in macrophages. When human monocyte-derived macrophages were cultured *in vitro* in the presence of clofazimine, the cells exhibited a marked decrease in metabolic activity and showed shrinkage in cell size, indicating cell death. Nuclear condensation and fragmentation were also observed by Giemsa and Hoechst 33248 stains. The endonuclease inhibitor $ZnCl_2$ inhibited the clofazimine-induced cell death. Significant enhancement of caspase-3 activity was observed in clofazimine-treated macrophages and THP-1 cells. Collectively, these results suggest the apoptosis-inducing activity of clofazimine in macrophages, which may also be responsible for the antibacterial properties of clofazimine.

Clofazimine (B663) is a phenazine iminoquinone derivative, specifically, a riminophenazine dye with the empirical formula $C_{27}H_{22}C_{12}$. In the 1950s, Barry et al. synthesized a large number of compounds by progressive chemical alteration of the anilinoaposafranine molecule, several of which showed antituberculous activity both *in vitro* and in experimental animals (1). Of these compounds, clofazimine (or Lamprene or B663) was found to be highly active against mycobacteria with the least toxicity. Chang (4) observed the antibacterial activity of clofazimine against *Mycobacterium lepraemurium* at about the same time as its anti-*M. leprae* activity was reported by Browne (2) and Browne and Hogenzeil (3). Later, after the introduction of the mouse footpad method of Shepard and Chang (22), its antibacterial activity against *M. leprae* was demonstrated (18).

Clofazimine has bifunctional activity: antibacterial and anti-inflammatory. It was used in the treatment of leprosy for its antibacterial action against *M. leprae*. Later, it was also found to possess an anti-inflammatory action which makes it a very useful drug in the treatment of acute reactions, including erythema nodosum leprosum (ENL), neuritis, iritis, etc., although its mechanism of action is unknown (2). *In vitro* studies on the effect of clofazimine on immune cells have been conducted. Clofazimine increases superoxide anion production and degranulation by stimulated neutrophils. and tumor necrosis factor alpha (TNF-α) potentiates this enhancement (15). The mechanism underlying this pro-oxidative effect seems to involve stimulation of phospholipase A2 (PLA2) activity with subsequent accumulation of arachidonic acid and lysophospholipids, which act as second messengers to activate oxidase (10). In addition, a number of reports have demonstrated the effects of clofazimine that might predict increased immune activity. Lysosomal enzyme activity of cultured macrophages was upregulated by clofazimine (21). Peripheral blood monocytes from healthy volunteers have been demonstrated to exhibit increased major histocompatibility complex class II expression following incubation with clofazimine (25). Increased oxygen uptake during phagocytosis was observed in neutrophils derived from patients with pyoderma gangrenosum during clofazimine therapy (5). Suppressor T-cell activity was decreased in mycobacteria-infected mice during clofazimine treatment (26). However, the mechanisms underlying the anti-inflammatory action of clofazimine are still unclear.

In the present study, we examined the effect of clofazimine on macrophages and found that the drug possessed apoptosis-inducing activity.

MATERIALS AND METHODS

Drug and chemicals. Clofazimine (Sigma-Aldrich Co., St. Louis, MO), rifampin (catalog no. R3501; Sigma-Aldrich Co.), and dapsone (DDS; Biomol Research Inc., Butler Pike Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −30°C until use. Ampicillin was obtained from Sigma-Aldrich Co.

Culture of human macrophages and isolation of bacilli. Human peripheral blood was obtained under informed consent from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) gradient centrifugation (12). The cells were suspended in AIM-V medium (Gibco BRL, Invitrogen Corp., Carlsbad, CA), and 1×10^6 PBMCs were cultured in a well of a 24-well tissue culture plate (Falcon; Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ) containing 13-mm round coverslips (Nunc Thermanox coverslips; Nalge Nunc, Thermo Scientific, Rochester, NY) at 37°C in a 5% CO_2 incubator for adherence of monocytes. After 1 h incubation, the coverslips were washed with Hanks' balanced salt solution (HBSS; Sigma-Aldrich Co.) to remove nonadherent cells. The monocytes on the coverslips were cultured in a new 24-well plate containing RPMI 1640 medium (Sigma-Aldrich Co.) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS; Bio Whittaker Co., Walkersville, MD), 2 mM L-glutamine, and 100 μg/ml ampicillin (RPMI-10F) in the presence of 40 ng/ml of granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN). After 10 days, the cells were differentiated into macrophages and used for experiments. In some experiments, PBMCs were cultured in 35-mm cell culture dishes (Corning Inc., Corning, NY) for adherence, and adherent monocytes were cultured for 10 days. Human monocytic leukemia cell line THP-1 was maintained in RPMI 1640 medium containing 15% fetal bovine serum.

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M. leprae (Thai-53 strain) was isolated from the footpads of BALB/c *nu/nu* mice that had been inoculated with *M. leprae* 8 months prior to isolation, and the bacillary number was enumerated according to the method of Shepard and Chang (22).

Light and phase-contrast microscopy. Macrophages on the coverslip were fixed with absolute methanol, followed by performing Giemsa stain (Wako Co., Japan). After they were mounted on a glass slide, the cells were observed under a light microscope (Optiphot-2; Nikon Co., Tokyo, Japan). Photographs were taken with a digital camera (Nikon F70s). Macrophages in 35-mm dishes were incubated in the presence of clofazimine and observed under a phase-contrast microscope (Olympus CKX41 with ×10- and ×20-objective lenses). Photographs were taken with an Olympus DP50 system. Image acquisition and data processing were done using the DP controller software.

Fluorescence microscopy. Fluorescence staining for DNA was employed. Macrophages were cultured in an 8-well chamber slide (Lab-Tek II chamber slide system; Nalge Nunc). The cells were incubated in the presence of clofazimine and subsequently fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS). Hoechst 33342 dye (Sigma-Aldrich Co.) in PBS was added to the wells at a final concentration of 10 μM, and the slide was incubated for 1 h at 37°C. The cells on the slide were observed under a fluorescence microscope (Olympus BX60 with a ×40-objective lens) equipped with an Olympus DP50 system. The digital images were processed with DP controller software.

Determination of cell death. Cell viability was determined using the colorimetric method (Cell Titer 96 aqueous nonradioactive cell proliferation assay; Promega Corp., Madison, WI). Briefly, cells in a 24-well plate were incubated in the presence of clofazimine in phenol red-free RPMI 1640 medium containing 10% FBS, followed by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt/phenazine methosulfate solution (formazan reagent). After 1 h incubation at 37°C, an aliquot of medium was transferred into a well of a 96-well plate, and the developed color was measured by a microplate reader at 490 nm. In addition, the activity of lactate dehydrogenase (LDH), released from dead cells into culture supernatants, was measured by a colorimetric assay (Cytotox 96 nonradioactive cytotoxicity assay; Promega Corp.). The color that developed in a sample incubated with LDH substrate was measured by a microplate reader at 490 nm (Vmax; Molecular Devices Corp., Sunnyvale, CA).

DNA electrophoresis. THP-1 cells or macrophages were harvested from the culture, and DNA was purified by a spin column method (E.Z.N.A. tissue DNA kits; Omega Bio-Tek, Norcross, GA). Briefly, 5×10^6 cells incubated in the presence of clofazimine were harvested, centrifuged at 2,000 rpm for 5 min, washed once with PBS, and resuspended in PBS. Protease was added, the mixture was heated at 65°C for 5 min, and buffer BL was added. After the mixture was heated at 70°C for 10 min, ethanol was further added. The mixture was applied to a HiBind spin column and centrifuged. DNA bound to the column was finally eluted, and the DNA preparation was subjected to electrophoresis in a 1% agarose gel, followed by ethidium bromide staining, and DNA was visualized by UV transillumination.

Western blotting. THP-1 cells or macrophages incubated with clofazimine were washed once with PBS(−) and lysed in lysis buffer (CelLytic-M; Sigma-Aldrich Co.) containing 2 protease inhibitor cocktails (phosphatase inhibitor cocktail 1 and phosphatase inhibitor cocktail 2; Sigma-Aldrich Co.). In the case of clofazimine-treated adherent macrophages, the cells were scraped off the dishes with a rubber policeman. The lysates were incubated for 10 min on ice and centrifuged at 13,000 rpm for 5 min. The protein concentration was determined. Ten micrograms of total protein was loaded onto an SDS-PAGE gel. After running the electrophoresis, the proteins in the gel were transferred onto an Immobilon PSQ membrane (Millipore Corporation, Billerica, MA). After washing with Tris-buffered saline (2.42 g Tris base and 8 g NaCl per 1 liter, pH 7.6) containing 0.05% Tween 20 (TBS-T), the membrane was blocked with 5% skim milk (Amersham ECL Plus Western blotting reagent pack; GE Healthcare Life Sciences, Amersham Place, Buckinghamshire, United Kingdom) for 1 h at room temperature. The membrane was washed 3 times with TBS-T and incubated overnight with 1:3,000-diluted primary anticleaved caspase-3 antibody (cleaved caspase antibody sampler kit; Cell Signaling Technology Inc., Danvers, MA). The membrane was then incubated with 1:10,000-diluted horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Finally, proteins were detected by incubating the membrane with HRP substrate (Immobilon Western chemiluminescent HRP substrate; Millipore Corporation), and the membrane was exposed to X-ray film (Amersham Hyperfilm ECL; GE Healthcare). For reprobing of the membrane, the membrane was washed with TBS-T and incubated with stripping buffer (Restore Plus Western blot stripping buffer; Pierce, IL). After the membrane was blocked, it was used again for probing different antibodies, such as cleaved caspase-9 and poly(ADP-ribose) polymerase (PARP) antibodies (cleaved caspase antibody sampler kit; Cell Signaling Technology Inc.) and beta-actin antibody (Cell Signaling Technology Inc.).

Colorimetric caspase assay. Colorimetric substrates for caspases were used to determine caspase-3 activity (colorimetric caspase assay kits; Biovision Research Products, CA) in lysates of cells incubated in the presence of clofazimine. Briefly, 5×10^6 cells were pelleted and lysed with chilled lysis buffer. After centrifugation, the supernatant was transferred to a new tube, and reaction buffer and a substrate for caspase-3, Asp-Glu-Val-Asp-*p*-nitroanilide, were added to the tube. After incubation for 2 h at 37°C, the samples were transferred into a well of a 384-well plate and read by a plate reader at 405 nm (Infinite F200; Tecan Systems Inc., San Jose, CA). The background reading was obtained by subtracting the reading for the reaction buffer from the reading for the lysate samples.

PGE_2 assay. The amount of prostaglandin E_2 (PGE_2) in the culture supernatants was measured by enzyme-linked immunosorbent assay (catalog no. 514010; Cayman Chemical Co., MI).

RESULTS

Morphological changes observed after treatment with clofazimine. Macrophages differentiated from human monocytes were incubated in the presence of 10 μg/ml of clofazimine for 20 h. The change in cell morphology was observed under a phase-contrast microscope. As shown in Fig. 1B, in the presence of clofazimine, the cells exhibited shrinkage in cell size and membrane blebbing. The death of more than 80% of cells was observed (Fig. 1B). As a control, Fig. 1A shows the normal morphology of macrophages. By Giemsa stain, too, these clofazimine-treated cells exhibited shrinkage in cell size, accompanied by the appearance of fragmented smaller nuclei (arrow in Fig. 1D), suggesting the apoptotic nature of the cells. Nontreated macrophages showed intact nuclei (arrow in Fig. 1C). Again, the change of nuclear structure was confirmed by Hoechst dye staining. Under a fluorescence microscope, nuclear condensation and membrane blebbing were observed in the clofazimine-treated cells (Fig. 1F and G), in contrast to normal cells, which showed intact nuclei (Fig. 1E). Similar fragmentation or condensation of chromatin was observed in THP-1 cells (data not shown). Such morphological changes were not observed in THP-1 cells treated with rifampin or dapsone at a concentration up to 50 μg/ml. Also, DMSO, which was used as a solvent for clofazimine at a concentration of 0.2%, had no effect on cell morphology or cell functions (negative control).

Cell death-inducing activity of clofazimine determined by colorimetric assay. Cell death was determined by a biochemical analysis using a colorimetric method. The conversion of the tetrazolium compound into soluble formazan is accomplished by metabolically active cells. When higher concentrations up to 10 μg/ml of clofazimine were employed in macrophage cultures, decreased color intensity of soluble formazan was observed, indicating cell death (Fig. 2A). Cell death was also observed in THP-1 cells (Fig. 2B). Hansen's disease is caused by infection of macrophages with *M. leprae*; therefore, we are curious to know whether *M. leprae* infection affects the cell death-inducing activity of clofazimine. When we infected macrophages with *M. leprae* at a multiplicity of infection (MOI) of 10 or 30, we found no significant difference in the induction of cell death in the presence of 10 μg/ml clofazimine, indicating that the bacilli did not inhibit or enhance clofazimine-induced cell death (Fig. 2C). Another method of determining cell death is by measurement of LDH release from

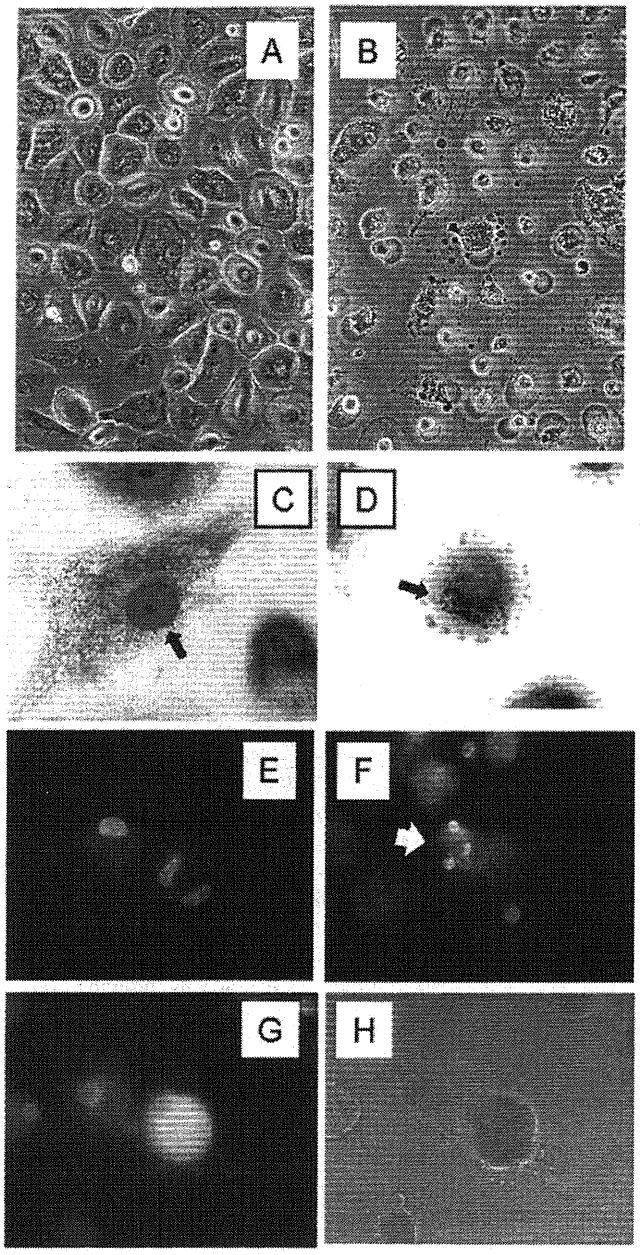

FIG. 1. Cell death induced in macrophages by clofazimine. Photographs were taken under a phase-contrast microscope (A and B) with a ×20-objective lens. (B) Human monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine for 20 h. (A) Cells cultured in the absence of clofazimine showed normal morphology. Giemsa stain of clofazimine-treated macrophages was also performed (C and D). Human monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine (D) or in the absence of clofazimine (C) for 24 h. Photographs were taken under a light microscope with a ×100-objective lens. Fragmentation of the nucleus was significant in the clofazimine-treated cells (arrow in panel D), in contrast to the intact morphology of the nucleus in normal cells (arrow in panel C). Nuclear condensation and fragmentation of clofazimine-treated macrophages were also confirmed under a fluorescence microscope (E to G). Macrophages were incubated in the presence of 10 μg/ml of clofazimine, followed by fixation and stained with a nucleus-staining dye, Hoechst 33342. The cells were observed under a fluorescence microscope (×40-objective lens). Cells cultured without clofazimine (E), clofazimine-treated cells (F and G), and a phase-contrast image of panel G (H) are shown.

dead cells. As shown in Fig. 3, more LDH release was observed in the manner dependent on the concentration of clofazimine.

Clofazimine treatment induces DNA ladder formation in macrophages. We examined the condition of DNA in clofazimine-treated THP-1 cells. Agarose gel electrophoresis showed fragmentation of DNA into integer multiples of 180 bp, a so-called DNA ladder (Fig. 4A), suggesting that DNA endonuclease was activated by clofazimine treatment. Therefore, we examined the effect of one of the apoptosis inhibitors, $ZnCl_2$, which is known to possess suppressing activity for endonuclease, and found that clofazimine-induced DNA fragmentation in THP-1 cells was completely blocked by $ZnCl_2$ treatment even at a low concentration of 0.25 mM $ZnCl_2$ (Fig. 4B), although it is still not clear whether $ZnCl_2$ can directly block the activity of clofazimine. Moreover, it was evident that neither cell death nor DNA fragmentation was induced by other antileprosy drugs, such as DDS or rifampin (Fig. 4C).

Clofazimine-induced cell death is mediated by activation of caspase-3. Caspases are known to be central regulators of apoptotic cell death, and caspase-3, which locates downstream of the caspase pathway, is one of the key executioners of apoptosis. Upon apoptotic stimulation, caspases are cleaved into active fragments. Figure 5 shows a Western blot analysis of extracts from THP-1 cells and macrophages cultured in the presence of clofazimine. Enhanced expression of cleaved caspase-3 was detected in cells (Fig. 5A and B). In addition, caspase-9 was also cleaved. A DNA-repairing enzyme, PARP, which is cleaved by caspase-3, was significantly activated in clofazimine-treated THP-1 cells (Fig. 5A). We next measured the caspase activity by colorimetric assay (Fig. 5C). The induction of caspase-3 by clofazimine was significantly high in macrophages as well as THP-1 cells.

Clofazimine enhanced PGE_2 production in *M. leprae*-infected macrophages. Monocyte-derived macrophages were preincubated in the presence of clofazimine for 4 h, followed by replenishment with *M. leprae*-containing medium for 20 h. The culture supernatants were collected, and the PGE_2 concentration was measured. As shown in Fig. 6, clofazimine clearly enhanced PGE_2 production in macrophages.

DISCUSSION

Riminophenazines are structurally phenazine compounds which were derived from lichens historically and were targeted for treatment of tuberculosis. The first clinically developed phenazine compound was clofazimine, whose activity has been extended to other mycobacterial diseases (1, 17). In test animals, the drug was found to inhibit the growth of mycobacteria *in vivo*, as well as *in vitro* (22), but the molecular mechanism of clofazimine in inducing anti-*M. leprae* activity is still not yet clear.

In the present study, it was found that both human monocyte-derived macrophages and THP-1 cells exhibited marked decreases in their metabolic activity in the presence of 10 μg/ml clofazimine. Under a phase-contrast microscope, 80% of the cells showed irregular morphology with shrinkage in cell size, and by a precise time course study, it was revealed that the morphological changes were evident from 6 h incubation with clofazimine. From this early time point, the cell body began to shrink, accompanied by membrane blebbing, which was also

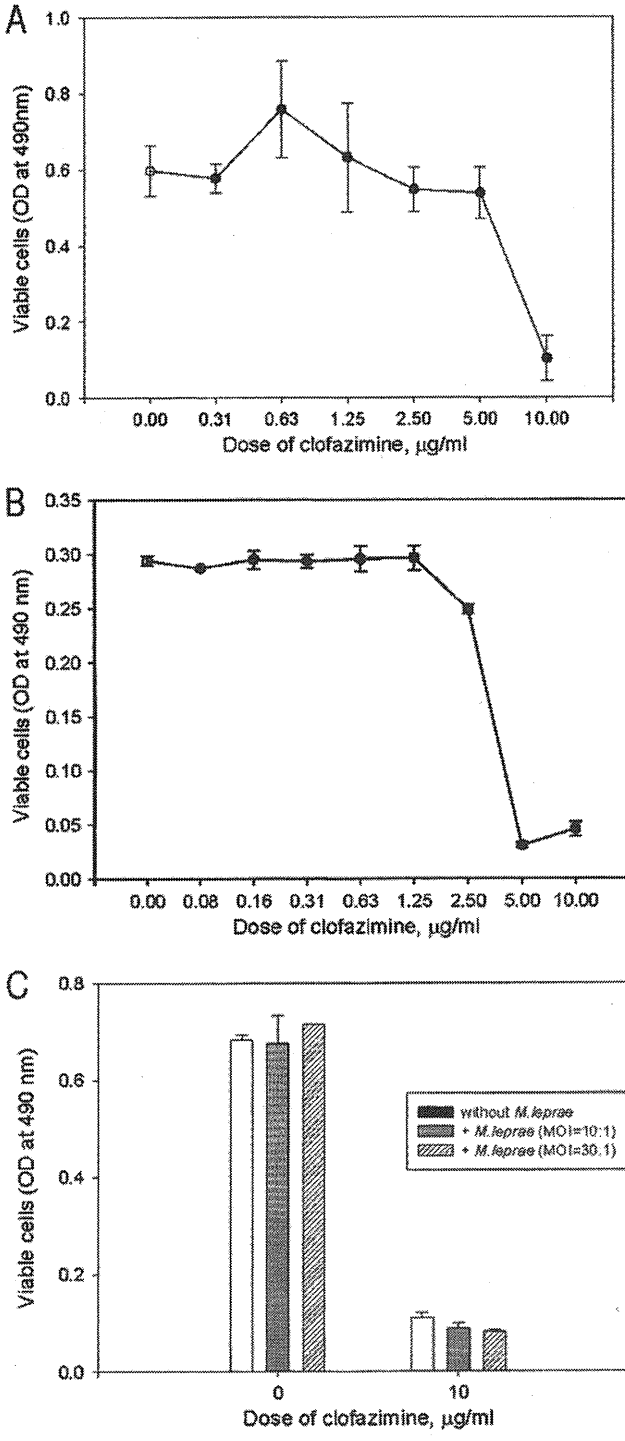

FIG. 2. Clofazimine-induced cell death in macrophages and THP-1 cells. Human monocyte-derived macrophages (A) and THP-1 cells (B) were incubated with various concentrations of clofazimine for 24 h, followed by determination of viable cells by the Cell Titer 96 cell proliferation assay. The results are representative of three independent cell culture tests. The cell death-inducing effect of clofazimine in the presence of *M. leprae* was also examined. Monocyte-derived macrophages were infected with *M. leprae* at an MOI of 10 or 30 per cell for 24 h. The infected cells were further incubated with 10 μg/ml clofazimine for another 24 h, followed by determination of viable cells by Cell Titer 96 cell proliferation assay (C). The results are representative of three independent cell culture tests. OD, optical density.

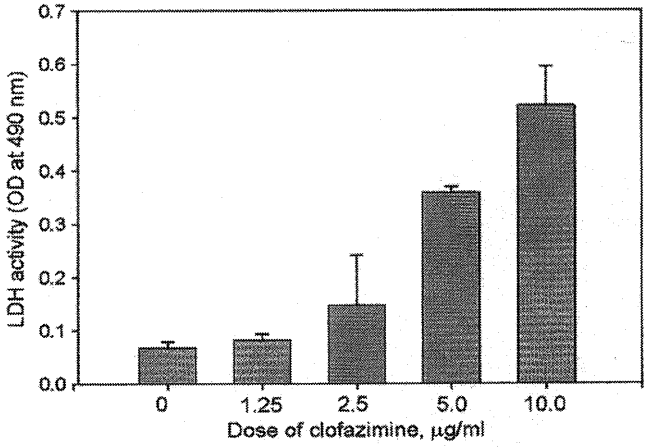

FIG. 3. LDH release from clofazimine-treated macrophages. Human monocyte-derived macrophages were incubated in the presence of the indicated concentrations of clofazimine for 24 h, and the LDH activity was measured. The results were obtained from triplicate cultures and are representative of three independent cell culture tests. OD, optical density.

evident from Giemsa stain and Hoechst staining of the nuclei (Fig. 1). Interestingly, the dose of clofazimine (10 μg/ml) required to cause cell death was equivalent to the dose required to exhibit anti-*M. leprae* activity *in vitro* by radiorespirometry (data not shown), the dose of which is in concordance with the dose required to kill *M. leprae* reported by Franzblau and O'Sullivan (7). Moreover, in our study, at 5-μg/ml concentrations of clofazimine, *M. leprae* viability was lowered in *in vitro* experiments with *M. leprae*-infected macrophages, and with this dose, *M. leprae* was found not to inhibit clofazimine-induced cell death. Therefore, clofazimine might inhibit mycobacterial growth through an alternative way by inducing apoptosis of host cells. Although the concentration of clofazimine in sera of patients taking regular doses of the drug is as low as 1 to 2 μg/ml, fat-soluble clofazimine readily accumulates in cells. In one patient, 7 months treatment with clofazimine (200 mg/day) resulted in accumulation of needle-shaped crystal inclusions in his alveolar macrophages (20). In another report, clofazimine-induced crystal-storing histiocytosis was observed in a leprosy patient (23). So, we are of the opinion that in some cells, the concentration of clofazimine is higher (10 to 20 μg/ml) than in others, so we have used a concentration of 10 μg/ml for our experiments.

Normally, cells undergo distinct morphological changes when they progress through either necrosis or apoptosis. Necrosis occurs when cells are exposed to an extreme variance from physiological conditions, resulting in damage to the plasma membrane. As such, necrosis is characterized by cell swelling and disruption of cellular organelles, with little change in the chromatin initially. In contrast, apoptotic cells shrink in size, undergo membrane blebbing, and exhibit marked alterations in their chromatin structure at an early stage under normal physiological conditions. As mentioned earlier, treatment with clofazimine resulted in highly condensed chromatin within the nucleus and membrane blebbing, indicating macrophages undergoing apoptosis. To confirm this, DNA from clofazimine-treated THP-1 cells was examined. Fragmented DNA was

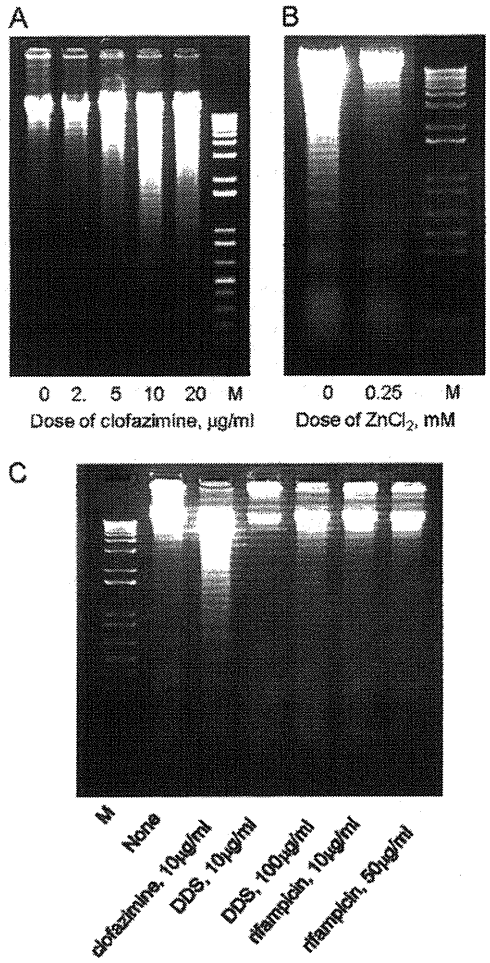

FIG. 4. DNA ladder formation in clofazimine-treated THP-1 cells and effects of other antileprosy drugs on DNA ladder formation. (A) THP-1 cells were incubated in the presence of the indicated concentrations of clofazimine for 4 h, followed by purification of DNA for agarose gel electrophoresis. An ethidium bromide-stained agarose gel is shown. (B) An endonuclease inhibitor, $ZnCl_2$, was examined for its effect on clofazimine-induced ladder formation. THP-1 cells were incubated in the presence of 10 μg/ml clofazimine and $ZnCl_2$ for 4 h. DNA was purified for electrophoresis. (C) THP-1 cells were incubated in the presence of clofazimine, DDS, and rifampin for 4 h, followed by purification of DNA. An ethidium bromide-stained agarose gel is shown. Lanes M, molecular weight marker.

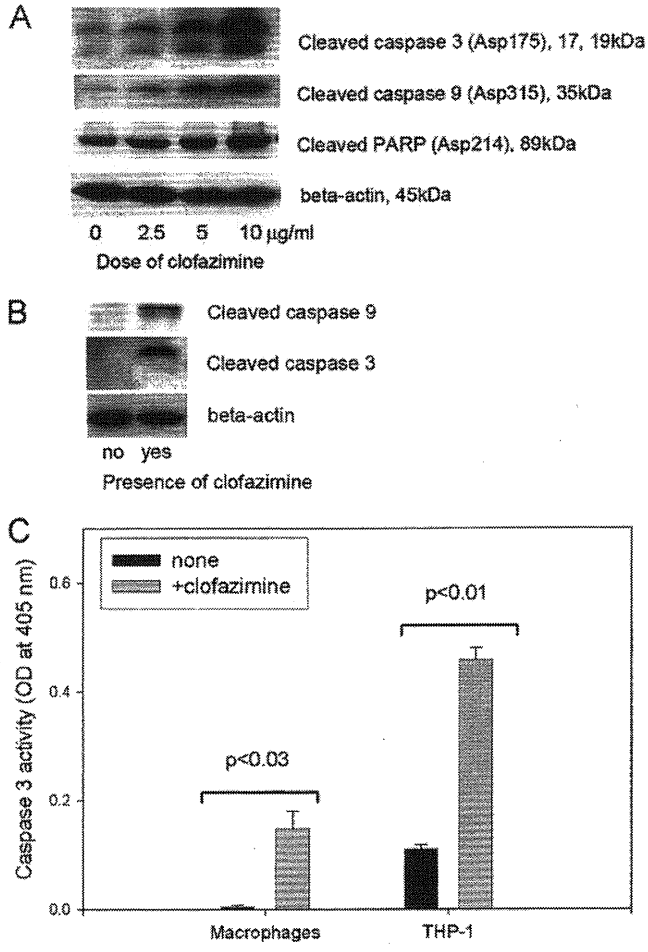

FIG. 5. Expression of caspase in clofazimine-treated THP-1 cells and macrophages. THP-1 cells were incubated in the presence of the indicated concentrations of clofazimine for 6 h, and cell lysates were processed for detection of cleaved caspase-3, caspase-9, and PARP by Western blotting (A). Similarly, monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine, and the cell lysates were examined for cleaved caspase-3 and caspase-9 expression (B). The caspase activity in clofazimine-treated macrophages and THP-1 cells was analyzed. Macrophages were incubated in the presence of 10 μg/ml of clofazimine for 6 h, and the caspase-3 activity in the cell lysates was determined by colorimetric assay (C). The results are representative of three independent cell culture tests.

demonstrated, suggesting that DNA endonuclease was activated causing apoptosis.

We observed that *M. leprae* by itself does not induce apoptosis of human cells. Similarly, infection of mouse macrophages with viable *M. leprae* was shown not to induce apoptosis (11). Although apoptosis is induced when macrophages infected with *M. leprae* are treated with clofazimine, the host cell viability does not change significantly in the presence of *M. leprae*. Nevertheless, the viability of *M. leprae* in macrophages was significantly lower in clofazimine-treated cells than infected cells not treated with clofazimine (data not shown). Therefore, we can speculate that clofazimine induces apoptosis of *M. leprae*-infected macrophages, which in turn inhibits *M. leprae* growth.

Consequently, we investigated the pathways involved in the execution of macrophage apoptosis (6, 14). We observed enhanced expression of cleaved caspase-3, caspase-9, and PARP following clofazimine treatment in THP-1 cells (Fig. 5A). Colorimetric assay also indicated enhanced caspase-3 activity in both macrophages and THP-1 cells treated with clofazimine (Fig. 5C), suggesting the involvement of caspases in clofazimine-induced apoptosis.

Apoptosis has been shown to be effective in therapy of chronic inflammatory diseases (16). An immunomodulatory drug, thalidomide, is used for treatment of ENL in leprosy patients, and its anti-inflammatory activity is believed to be through the downregulation of production of the proinflammatory cytokine TNF-α (19). Gockel et al. showed that thalidomide induces apoptosis in human monocytes (8). Clofazimine

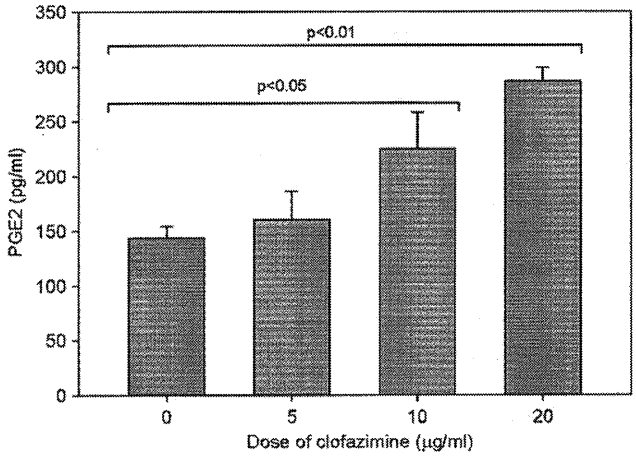

FIG. 6. Enhancement of PGE_2 production by clofazimine in *M. leprae*-infected macrophages. Macrophages were preincubated in the presence of the indicated doses of clofazimine for 4 h, followed by infection with *M. leprae* (5×10^6/well), and the culture was continued for another 20 h. The amount of PGE_2 in the culture supernatants was measured. The results are representative of three independent cell culture tests.

is known to have a favorable influence on the reversal reaction in human leprosy (2). Browne and Hogenzeil found that clofazimine controlled persistent exacerbations in patients who were corticosteroid dependent for controlling the passing reactions, including ENL, and suggested that the drug may exert a suppressive effect on the development of acute exacerbation in lepromatous leprosy (3). These observations have been followed by those of later workers, and one of the special indications for use of clofazimine is the presence of acute reactions or a chronic recurrent reaction in lepromatous leprosy. These clinical data suggest that the mechanisms underlying the action of the drug in these leprosy patients mainly seem to be anti-inflammatory, although there is no direct evidence. Macrophages are capable of elaborating a series of biochemical products with potent immunomodulatory activities. We have observed enhancement of the production of PGE_2, when macrophages were pretreated with clofazimine. PGE_2 is released from arachidonic acid by PLA2. The enzyme is reported to be stimulated in clofazimine-treated neutrophils (10). With respect to B-cell function, prostaglandins of the E series (PGE) inhibit both B-cell proliferation and the generation of antibody-forming cells, and also, B-cell tolerance is induced by PGE_2 (9, 24). T-cell proliferation is also suppressed by PGE_2 (13). The reaction to leprosy involves antibody (ENL caused by immune complex) and cells (delayed-type hypersensitivity mediated mainly by T cells). The mechanisms underlying the immunomodulatory role of clofazimine are still not clear, but the present study clarifies certain aspects. Apoptosis induced in macrophages might explain the anti-inflammatory activities of clofazimine *in vivo*.

In conclusion, our findings suggest that clofazimine induced apoptosis of macrophages through the activation of caspases. The data indicate that the action of clofazimine in leprosy patients may be at least partially mediated by apoptosis.

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Synthesis and evaluation of anti-tubercular activity of new dithiocarbamate sugar derivatives

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ABSTRACT

The present study was undertaken to optimize the anti-tubercular activity of 2-acetamido-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (OCT313, Glc-NAc-DMDC), a lead compound previously reported by us. Structural modifications of OCT313 included the replacements of the DMDC group at C-1 by pyrrolidine dithiocarbamate (PDTC) and the acetyl group at C-2 by either propyl, butyl, benzyl or oleic acid groups. The antimycobacterial activities of these derivatives were evaluated against *Mycobacterium tuberculosis* (MTB). Glc-NAc-pyrrolidine dithiocarbamate (OCT313HK, Glc-NAc-PDTC) exhibited the most potent anti-tubercular activity with the minimal inhibitory concentration (MIC) of 6.25–12.5 μg/ml. The antibacterial activity of OCT313HK was highly specific to MTB and *Mycobacterium bovis* BCG, but not against *Mycobacterium avium*, *Mycobacterium smegmatis*, *Staphylococcus aureus* or *Escherichia coli*. Importantly, OCT313HK was also effective against MTB clinical isolates, including multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. Interestingly, OCT313HK was exerted the primary bactericidal activity, and it was also exhibited the bacteriolytic activity at high concentrations. We next investigated whether the mycobacterial monooxygenase EthA, a common activator of thiocarbamide-containing anti-tubercular drugs, also activated OCT313HK. Contrary to our expectations, the anti-tubercular activity of dithiocarbamate sugar derivatives and dithiocarbamates were not dependent on *ethA* expression, in contrast to thiocarbamide-containing drugs. Overall, this study presents OCT313HK as a novel and potent compound against MTB, particularly promising to overcome drug resistance.

More than 9.4 million people develop tuberculosis (TB) annually, and 1.7 million die each year.[1] New case of TB is still increasing all over the world, especially in low-income countries, and TB infection including both multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) is a leading cause of death worldwide. The spread of both MDR-TB and XDR-TB, due to poor compliance of anti-TB drugs, becomes a global health problem. Forty years have passed since the last development of anti-TB drug and the development of novel and innovative compounds is urgently needed.[2] We have recently reported that 2-acetamido-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (Glc-NAc-DMDC), named as OCT313, exhibited the potent antimycobacterial activity.[3]

Studies on the structure–activity relationships (SAR) at C-1, C-4 and C-6 positions of OCT313 established that the DMDC group at C-1 position was critical to the bactericidal activity. In this study, in order to improve the antimycobacterial activity of OCT313, we synthesized the derivative of dithiocarbamate group at the C-1 position and its antimycobacterial activity was evaluated. We first examined whether the elongation of alkyl side chain of dimethyldithiocarbamate, for example, diethyl and dibutyl, improved the antimycobacterial activity against *Mycobacterium tuberculosis* (MTB) $H_{37}Rv$. The elongation of carbon chain resulted in decreasing of anti-tubercular activity (Table 1). Previously, the antimycobacterial activity of pyrrolidine dithiocarbamate (PDTC) and dialkyldithiocarbamate derivatives have been demonstrated.[4–6] Next, we investigated whether dithiocarbamates containing heterocyclic ring, for example, 4-imidazodithiocarboxylic acid (IMTC) and PDTC were effective against MTB. As a result, PDTC was the most potent compound in our experiments, which was similar to first-line drugs in vitro.

Based on these findings, we synthesized C-1 derivative of OCT313, 2-acetamido-2-deoxy-β-D-glucopyranosyl pyrrolidine-1-

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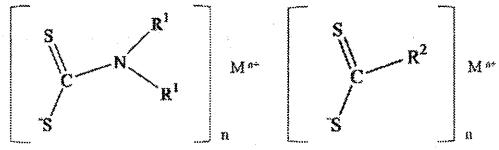
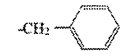
Table 1
Antimycobacterial activity of dithiocarbamate derivatives



Agent	*n*	M	R^1	R^2	MIC for (MIC, μg/ml)		
					M. tuberculosis $H_{37}Rv$	*M. bovis* BCG str. Tokyo 172	*M. smegmatis* JATA 64-01
Carbon chain							
DMDC	1	Na	$-CH_3$	–	1.56	1.56	>100
DDC	1	Na	$-CH_3CH_3$	–	3.13	3.13	>100
DDC	2	Zn	$-CH_2CH_3$	–	1.56	1.56	>100
DBuDC	2	Zn	$-CH_2CH_2CH_2CH_3$	–	12.5	12 5	>100
Aromatic ring							
DBzDC	2	Zn			25	25	>100
Heterocyclic ring							
IMTC	1	Na			6.25	12 5	>100
PDTC	1	NH_3			0.2	0.4	>100

DMDC. Na, sodium dimethyldithiocarbamate; DDC. Na, sodium diethyldithiocarbamate; DDC. Zn, zinc bis (diethyldithiocarbamate); DBuDC. Zn, zinc bis(dibutyldithiocarbamate); DBzDC. Zn, zinc bis(dibenzyldithiocarbamate); IMTC. Na, sodium 4-imidazodithiocarboxylic acid; PDTC. NH_3, ammonium 1-pyrrolidine dithiocarbamate.

carbodithioate (OCT313HK, Glc-NAc-PDTC),[14,15] which is the substitution of the DMDC group at C-1 position of OCT313 to the PDTC group and was determined the antibacterial activity (Table 2). OCT313HK exhibited the potent antimycobacterial activity against both MTB and *Mycobacterium bovis* BCG Tokyo with MICs of 6.25 μg/ml and 12.5 μg/ml, respectively (Table 2). However, OCT313HK failed to inhibit the growth of *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium* and *Pseudomonas aeruginosa*. (Table 2 and Supplementary Table 1). Meanwhile, PDTC exhibited the anti-tubercular activity (MIC = 0.2 μg/ml) and antibacterial activities against *S. aureus* (MIC = 8–12.5 μg/ml), *E. faecalis* (MIC = 32 μg/ml), *E. faecium* (MIC = 32 μg/ml), but not *P. aeruginosa* (Table 2 and Supplementary Table 1). These data indicate that the antibacterial spectrum of OCT313HK is narrow compared to PDTC and exhibit 2 to 4-fold higher anti-tubercular activity than OCT313 (MIC = 25 μg/ml, Table 2).

We next evaluated the antimycobacterial activities of OCT313HK and PDTC against 40 MTB clinical isolates, including drug-sensitive and drug-resistant strains. As shown in Table 3, OCT313HK exhibited the anti-tubercular activity with the MIC of 6.25–12.5 μg/ml, whereas the MIC value of PDTC was 0.2–0.4 μg/ml. Importantly, no cross resistance to almost currently used anti-TB drugs was demonstrated, that OCT313HK and PDTC exhibited comparable activities against all these strains, including five

Table 2
Antibacterial activity of OCT313HK in vitro (MIC, μg/ml)[a]

Compound	Organisms							
	M tuberculosis $H_{37}RV$	*M. bovis* BCG str. Tokyo 172	*M. avium* subsp. hominissuis 104	*M. avium* subsp. avium ATCC25291	*M. smegmatis* JATA 64-01	*S. aureus*	MRSA 873	*E. coli* DH5α
Synthetic derivative								
OCT313HK	6.25	12.5	100	50	>100	>100	>100	>100
OCT313	25	31.3	>100	>100	>100	>100	>100	>100
Raw material								
Glc-NAc free	>100	>100	>100	>100	>100	>100	>100	>100
DMDC. Na	0.78	1.56	>100	>100	>100	>100	>100	>100
PDTC. NHi	0.2	0.4	3.13	3 13	100	12.5	12.5	12.5
anti-IB drug								
INH	0.04	0.04	1.56	3 13	6.25	>100	>100	>100
RFP	0.004	0.004	0.25	<0.05	1.56	0.002	0.004	50
SM	0.39	0.2	1.56	3,13	0.39	50	>100	50
EB	2.5	1.5	1.6	1.6	12.5	>100	>100	>100
KM	1.56	0.3	3.13	3.13	3.13	12.5	>100	12.5
CPFX	0.39	0.1	0.39	1.56	0.39	0.2	ne	0.2
β-Lactam antibiotics								
PCG	500	500	ne	ne	ne	31.3	>500	25
ABPC	12.5	12.5	ne	ne	ne	50	>100	>100
IPM	3.13	3.13	ne	ne	ne	0.1	0.1	0.1

[a] Broth dilution methods using MiddleBrook 7H9 broth containing albumin, dextrose, and catalase for derivatives (ne, not examined). For *Staphylococcus aureus*, we used the LB broth. OCT313HK, Glc-NAc-PDTC; OCT313, Glc-NAc-DMDC, Glc-NAc free, *N*-acetyl glucosamine; DMDC. Na, sodium dimethyldithiocarbamate; PDTC. NH_3, ammonium 1-pyrrolidine dithiocarbamate; INH, isoniazid; RFP, rifampicin, SM, streptomycin; EB, ethambutol; KM, kanamycin, PAS, para-aminosalicylic acid; CPFX, ciprofloxacin; PCG, penicillin G; ABPC, aminobenzyl penicillin; IPM, imipenem.

Table 3
Antimycobacterial activities of OCT313HK and PDTC against clinical isolates of *M. tuberculosis*

Clinical isolates	Resistance to	MIC for (μg/ml)	
		OCT313HK	PDTC
Drug sensitive strain			
1		6.25	0.2
2		6.25	0.2
3		6.25	0.2
4		6.25	0.2
5		6.25	0.2
6		6.25	0.2
7		12.5	0.4
8		12.5	0.4
9		6.25	0.2
10		6.25	0.2
11		6.25	0.2
12		6.25	0.2
13		12.5	0.4
14		6.25	0.2
15		6.25	0.2
16		6.25	0.2
17		6.25	0.2
18		6.25	0.2
19		6.25	0.4
20		12.5	0.4
Drug-resistant strain			
1	INH, RFP, EB, LVFX, SPFX, CPFX	6.25	0.2
2	INH, RFP, EB, LVFX, SPFX, CPFX	6.25	0.2
3	RFP, EB	6.25	0.2
4	INH, RFP, EB, LVFX, SPFX, CPFX	12.5	0.4
5	INH, RFP, EB, KM, LVFX, SPFX, CPFX	6.25	0.2
6	INH, RFP, SM, EB, KM, LVFX, SPFX, CPFX	6.25	0.2
7	RFP	6.25	0.2
8	RFP	6.25	0.2
9	INH, RFP, KM, LVFX, SPFX, CPFX	6.25	0.2
10	RFP	12.5	0.4
11	INH, RFP, EB, KM, LVFX, SPFX, CPFX	12.5	0.2
12	INH, RFP, EB	6.25	0.2
13	INH, RFP, SM, EB	6.25	0.2
14	INH, RFP, EB	6.25	0.2
15	INH, RFP, EB, LVFX, SPFX, CPFX	6.25	0.2
16	INH, RFP, SM, EB, KM, PAS, EVM, LVFX	6.25	0.2
17	INH, RFP	6.25	0.2
18	INH, RFP, SM, EB, PAS	6.25	0.2
19	INH, RFP, SM, EB, PAS, LVFX	12.5	0.4
20	INH, RFP, ETH	6.25	0.2
H_3-Rv		6.25–12.5	0.2–0.4

OCT313HK, Glc-NAc-PDTC; PDTC, ammonium 1-pyrrolidine dithiocarbamate; INH, isoniazid; RFP, rifampicin; SM, streptomycin; EB, ethambutol; KM, kanamycin; PAS, *para*-aminosalicylic acid; ETH, ethionamide; EVM, emviomycin; LVFX, levofloxacin; SPFX, sparfloxacin; CPFX, ciprofloxacin.

XDR-TB strains. XDR-TB is defined as TB that is resistant to at least rifampicin and isoniazid plus fluoroquinolones, and at least one of three injectable second line anti-TB drugs, that is, amikacin, kanamycin or capreomycin.[1] These results suggest that OCT313HK and PDTC are effective against strains, resistant to fluoroquinolones, e.g. levofloxacin (LVFX), sparfloxacin (SPFX), ciprofloxacin (CPFX), which have been considered as candidate compounds for new anti-TB therapy. Therefore, OCT313HK and PDTC may represent attractive drug candidates to be included in future pharmacological developments against XDR-TB.

Next, we investigated the primary mode of action of OCT313HK and OCT313. Each compound reduced not only the colony forming units, but also the optical density (OD) at both log-phase and stationary phase in time-rather than dose-dependent manner (Fig. 1 and Table 4). Both OCT313HK and OCT313 remarkably decreased the turbidity compared to other bactericidal drugs, that is, isoniazid (INH), ethionamide (ETH), streptomycin (SM), and kanamycin (KM) (data not shown). Consequently, we determined whether the lytic activity was suppressed by the presence of either dextran or sucrose, which was used to increase the extracellular osmotic pressure.[7] The lytic activity of OCT313HK and OCT313 against *M. bovis* BCG were inhibited in the presence of these reagents from day 2 (Fig. 1A and B). Taken together, these results suggest that OCT313HK and OCT313 exert bactericidal and bacteriolytic activities. Of note, this feature has not been observed with the currently used anti-TB drugs.

PDTC and DMDC belong to dithiocarbamates. Thiocarbamide-containing drugs, for example, ETH, thiacetazone (TAC) or isoxyl (ISO), which have been used as second line drugs are activated by the monooxygenase EthA.[8] Approximately, 50% of ETH-resistant clinical isolates possessed mutations in the *ethA* gene.[9] Thereby, we further studied whether *ethA* expression was required for antimycobacterial activity of dithiocarbamates and dithiocarbamate sugar derivatives. This was achieved by using *M. bovis* BCG strains carrying either the pMV261-*ethA* or the pMV261-*ethR*, designed to overexpress either EthA or EthR under the control of the constitutive *hsp60* promoter, respectively.[8] The MICs against these strains were compared to those of a BCG strain harboring the empty construct (Table 5). The EthR-overexpressing strain expressed high levels of resistance to ETH, whereas the EthA-overexpressing strain was hypersusceptible to ETH, consistently with previous reports.[8,10] In contrast, the MICs of dithiocarbamate-containing agents OCT313HK, OCT313 and PDTC against the EthA- or EthR-overexpressing strains were similar to those of the control strain, indicating that the anti-tubercular activity of these two compounds does not rely on EthA expression. In addition, OCT313HK and PDTC demonstrated no cross-resistance to ETH, because they were effective against ETH-resistant clinical isolate No. 20 (Table 3). These results suggest that the mode of action of both dithiocarbamate sugar derivatives and dithiocarbamates are different from the currently used anti-tubercular drugs, including ETH.

Finally, in order to study the SAR for sugar moieties of OCT313, we further synthesized the chemically modified derivatives at C-2 position of OCT313 (Table 6).[14,16] Previously, it was demonstrated that C-4 isomers of OCT313 was more potent anti-tubercular activity compared to OCT313. Nevertheless, the anti-tubercular activity of C-2 derivatives, which were modified to some other types of functional groups namely propionamido (R^2), butyramido (R^3), benzamido (R^4) and oleamido (R^5) were lower than original compound OCT313 (MIC = 50–100 μg/ml). Contrary to the strategy, 2-amino derivative of OCT313 (Glc-NH_2-DMDC) (R^6) was synthesized by de-*O*-acetylation of 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate hydrochloride with an anion exchange resin.[17] The anti-tubercular activity of Glc-NH_2-DMDC was similar to C-2 derivatives (MIC = 50 μg/ml). These results suggest that the acetyl group at C-2 position of OCT313 was optimal for anti-tubercular activity.

In conclusion, this study has unraveled the potential of OCT313HK and OCT313 as valuable compounds for future pharmacological developments against MDR-TB and XDR-TB. Interestingly, OCT313HK exhibited unstained bacteriolytic activity compared to OCT313. The lytic activity of dithiocarbamate sugar derivatives is probably due to dithiocarbamate structure. Surprisingly, dithiocarbamate sugar-resistant colonies were unable to grow on 7H11 agar plate whereas, both anti-TB drug- and dithiocarbamate-resistant colonies were observed spontaneously (data not shown). The resistant strains to anti-TB drug, for example, INH, RFP, SM, KM, PAS and CPFX, can be subcultured in liquid medium, commonly. Nevertheless, we were not able to subculture the resistant strains to dithiocarbamate, for example, PDTC and DDC, in any broth containing each agent. This phenomenon was caused by robust clumping of dithiocarbamate-resistant bacilli compared to other strains. Actually, the colonization and morphology of dithiocarbamate-resistant strains

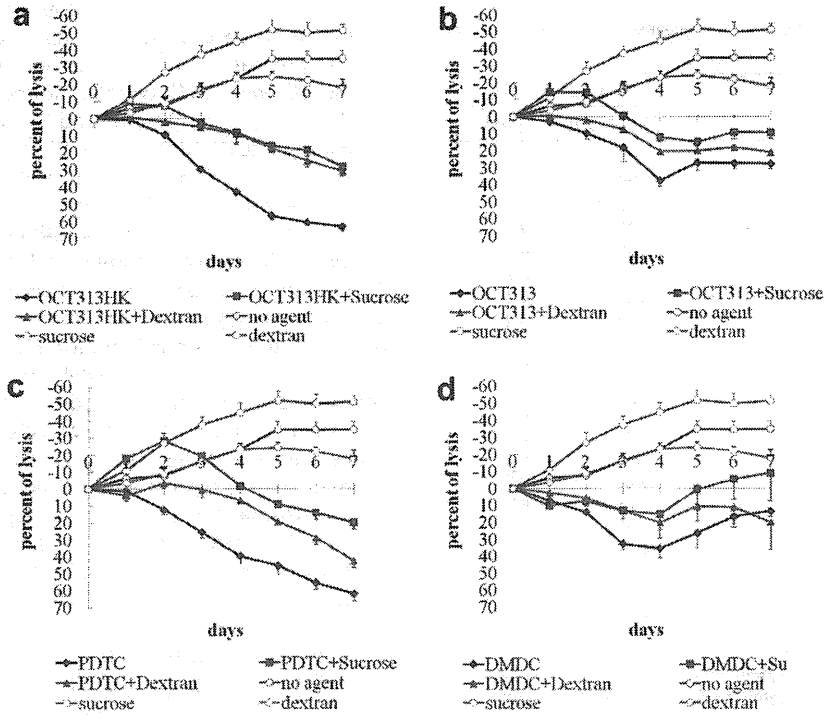


Figure 1. Bacteriolytic activity of each agent under the presence of either dextran or sucrose at log phase. Lysis of *M. bovis* BCG was determined by incubation with 125 mg/m OCT313HK (A), 313 mg/ml OCT313 (B), 4 mg/ml PDTC (C) and 15.6 mg/ml DMDC (D) with or without 150 mM sucrose or 2.5% dextran (molecular weight = >500,000) fo 7 days. Lysis of dilute suspensions of cells was determined by following spectrophotometrically the loss of reading at O.D. 530 nm. Experiments were carried out more tha three times and representative data were shown. Error bars represent means ± SD (n = 3).

Table 4
Bactericidal activity of each agent under in the presence of either dextran or sucrose at both log phase and stationary phase

	Agent and condition	Ave. log CFU/ml ± SD[a]	
		day 7	day 21
A			
	OCT313HK	4.55 ± 0.14	4.43 ± 0.08
	OCT313HK + sucrose	6.25 ± 0.03	5.76 ± 0.10
	OCT313HK + dextran	6.07 ± 0.13	5.79 ± 0.05
B			
	OCT313	5.91 ± 0.07	5.20 ± 0.14
	OCT313 + sucrose	6.41 ± 0.08	5.78 ± 0.04
	OCT313 + dextran	6.44 ± 0.08	5.91 ± 0.03
C			
	PDTC	4.65 ± 0.08	4.41 ± 0.08
	PDTC + sucrose	6.05 ± 0.03	5.56 ± 0.04
	PDTC + dextran	6.93 ± 0.06	5.56 ± 0.09
D			
	DMDC	6.94 ± 0.05	8.21 ± 0.23
	DMDC + sucrose	6.18 ± 0.07	8.22 ± 0.03
	DMDC + dextran	6.09 ± 0.04	8.12 ± 0.09
E			
	No agent	8.44 ± 0.35	7.92 ± 0.06
	Sucrose	8.50 ± 0.23	8.13 ± 0.06
	Dextran	8 34 ± 004	8.26 ± 004

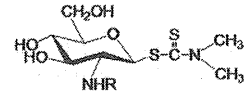
[a] Bactericidal activity against *M. bovis* BCG was determined by incubation with 125 μg/ml OCT313HK (A), 313 μg/ml OCT313 (B), 4 μg/ml PDTC (C) and 15.6 μg/ml DMDC (D) with or without 150 nM sucrose or 2.5% dextran for 7 days or 21 days, respectively. Values represent means ± SD. OCT313HK, Glc-NAc-PDTC; OCT313. Glc-NAc-DMDC; PDTC, ammonium 1-pyrrolidine dithiocarbamate; DMDC, sodium dimethyldithiocarbamate.

Table 5
MICs of ethionamide and dithiocarbamate-containing drugs against *M. bovis* BC overexpressing either EthA or EthR in 7H11 agar supplemented with OADC

Strain	MIC for (MIC, μg/ml)			
	OCT313HK	OCT313	PDTC	ETH
BCG pMV261	1–2.5	10–20	0.5	1–5
BCG pMV261 ::*ethA*	1–2.5	10–20	0.5	0.5
BCG pMV261::*ethR*	1–2.5	10–20	0.25–0.5	10–25

OCT313HK, Glc-NAc-PDTC; OCT313. Glc-NAc-DMDC; PDTC, ammonium 1-pyrroli dine dithiocarbamate; ETH. ethionamide.

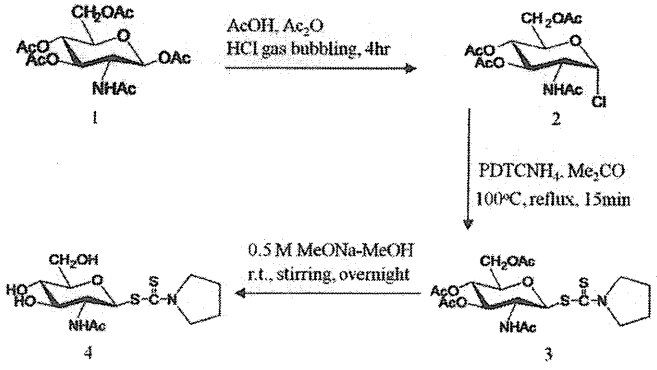
Table 6
Anti-tubercular activity of C2-derivatives of OCT313 in vitro[a]



R	Compound	MIC for (MIC, μg/ml) *M. tuberculosis* H_{37}-Rv
$R^1 = COCH_3$	OCT313 (Glc-NAc-DMDC)	25
$R^2 = COC_2H_5$	Glc-NPro-DMDC	50
$R^3 = COC_3H_5$	Glc-NBt-DMDC	50
$R^4 = COC_6H_6$	Glc-NBz-DMDC	100
$R^5 = COC_{17}H_{33}$	Gk-NOle-DMDC	100
R^6=H	Glc-NH_2-DMDC	50

[a] Broth dilution methods using MiddleBrook 7H9 broth containing albumin, dextrose, and catalase for derivatives.

were remarkably different from anti-TB drug-resistant strains. These findings suggested that the anti-tubercular activity of dithiocarbamate and dithiocarbamate sugar might be not caused due to some common known mechanism. Therefore, further work is required to clarify the specific targets of dithiocarbamate and dithiocarbamate sugar.



Scheme 1. Synthesis of 2-acetamido-2-deoxy-2-deoxy-β-D-glucopyranosyl pyrrolidine-1-carbodithioate (OCT313HK, Glc-NAc-PDTC) (**4**).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.084.

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14. *General procedures*: Melting points were determined with a Yamagimoto MP-S2 micro melting point apparatus and uncorrected. Solutions were concentrated in a rotary evaporator below 50 °C under vacuum. Optical rotations were measured with a JASCO P-1020 automatic digital polarimeter in a 0.1 dm tube. IR spectra were recorded with a JASCO FT/IR-4100 Spectrometer. ^{1}H NMR spectra were recorded at 500 MHz with a JNM-α500 spectrometer and JNM-ECA500/KJ, at 600 MHz with a BRUKER-AV600. ^{13}C NMR spectra were recorded at 125 MHz with a JNM-α500 spectrometer. Tetramethylsilane was used as an internal standard. Chemical shift are given on the δ scale. TLC was performed on precoated silica gel plates 0.25 mm thick (Kieselgel 60F_{254}, Merck). Detection was effected with H_2SO_4 or by UV irradiation at 254 nm. Column chromatography was performed on Silica Gel BW-820MH (Fuji-Silysia Chemical Ltd, Nagoya, Japan).
15. 2-Acetamido-2-deoxy-β-D-glucopyranosyl pyrrolidine-1-carbodithioate (**4**) (Scheme 1) was synthesized as follows. Reaction of literature known 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[11] and ammonium 1-pyrrolidinecarbodithioate in acetone gave thioglycoside peracetate in 51.4% yields. In its ^{1}H NMR spectrum one proton doublet of H-1 appeared at δ 5.72 ($J_{1,2}$ = 11.0 Hz), indicative of β-configuration. De-*O*-acetylation of thioglycoside peracetate with 0.5 M sodium methoxide-methanol gave compound (2-acetamido-2-deoxy-β-D-glucopyranosyl pyrrolidine-1-carbodithioate) as colorless needles, mp 198–199° (decomp.) in 78% yields. $[\alpha]_D^{23}$ +46.4° (*c* 1.04, H_2O), IR(KBr) cm^{-1}: 3500–3300 (br OH, NH), 1639 (amide I), 1532 (amide II). ^{1}H NMR (CD_3OD) δ: 1.94 (s, 3H, $NCOCH_3$), 1.97, 2.07, 3.59, 3.70, 3.86 (m, 8H, methylene protons of pyrrolidine group), 3.38 (ddd, 1H, $J_{4,5}$ = 9.7 Hz, $J_{5,6a}$ = 5.0 Hz, $J_{5,6b}$ = 2.3 Hz, H-5), 3.42 (dd, 1H, $J_{3,4}$ = 8.6 Hz, H-4), 3.54 (dd, 1H, $J_{2,3}$ = 9.8 Hz, H-3), 3.68 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, H-6a), 3.82 (dd, 1H, H-6b), 4.05 (dd, 1H, $J_{1,2}$ = 11.0 Hz, H-2), and 5.72 (d, 1H, H-1). ^{13}C NMR(CD_3OD) δ 22.9 ($COCH_3$), 25.1, 26.9, 52.0, 56.3 (methylene carbons of pyrrolidine group), 54.5 (C-2), 62.6 (C-6), 71.6 (C-4), 77.6 (C-3), 82.3 (C-5), 89.4 (C-1), 173.6 (C=O), and 195.4 (C=S). HR-FAB-MS: *m/z* 351.1046 $[M+H]^+$ (calcd for $C_{13}H_{23}O_5N_2S_2$: 351.1049). 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl pyrrolidine-1-carbodithio ate (**3**): $[\alpha]_D^{25}$ +50.8° (*c* 1.06, $CHCl_3$), IR (KBr) cm^{-1}: 3283 (NH), 1747 (C=O), 1667 (amide I), 1544 (amide II). ^{1}H NMR($CDCl_3$) δ: 1.92 (s, 3H, $NCOCH_3$), 1.99, 2.05, 3.60, 3.73, 3.90 (m, 8H, methylene protons of pyrrolidine group), 2.05 (×2), 2.08 (s, 9H, $COCH_3$×3), 3.86 (ddd, 1H, $J_{4,5}$ = 9.8 Hz, $J_{5,6a}$ = 2.1 Hz, $J_{5,6b}$ = 4.9 Hz, H-5), 4.13 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.25 (dd, 1H, H-6b), 4.55 (ddd, 1H, $J_{1,2}$ = 11.0 Hz, $J_{2,NH}$ = $J_{2,3}$ = 9.8 Hz, H-2), 5.16 (dd, 1H, $J_{3,4}$ = 9.5 Hz, H-4), 5.21 (dd, 1H, H-3), 5.86 (d, 1H, H-1), and 6.25 (d, 1H, NH). ^{13}CNMR($CDCl_3$) δ 20.7 (×2), 20.8 ($OCOCH_3$×3), 23.2 ($NCOCH_3$), 24.2, 26.0, 51.1, 55.4 (methylene carbons of pyrrolidine group), 52.0 (C-2), 62.0 (C-6), 68.0 (C-4), 74.6 (C-3), 76.5 (C-5), 88.0 (C-1), 169.3, 170.2, 170.8, 171.2 ($NCOCH_3$, $OCOCH_3$×3), and 189.2 (C=S).
16. Acetamido group of OCT313 was chemically modified to some other types of functional groups namely propionamido (R^2), butyramido (R^3), and benzamido (R^4), oleamido (R^5) (Table 6). Synthetic method was as follows. After acyl chlorides or anhydride were reacted to literature known 1,3,4,6-tetra-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranose hydrochloride[12] resulting *N*-acyl-β-acetates were chlorinated with HCl gas in acetic acid and anhydride, and reacted with sodium *N,N*-dimethyldithiocarbamate in acetone. After de-*O*-acetylation some *N*-acyl derivatives of OCT313 were obtained. 2-Deoxy-2-propionamido-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (R^2): $[\alpha]_D^{22}$ +53.9° (*c* 1.11, MeOH), IR (KBr) cm^{-1}: 3510–3100 (br OH, NH), 1641 (amide I), 1571 (amide II). ^{1}H NMR (CD_3OD + D_2O, 3:4, v/v) δ: 1.11 (t, 3H, *J* = 7.6 Hz, CH_2CH_3), 2.24 (m, 2H, CH_2CH_3), 3.38, 3.53 (s, 6H, NCH_3×2), 3.49 (m, 1H, H-4), 3.50 (m, 1H, H-5), 3.64 (dd, 1H, $J_{2,3}$ = 9.9 Hz, $J_{3,4}$ = 8.7 Hz, H-3), 3.73 (dd, 1H, $J_{5,6a}$ = 0.6 Hz, $J_{6a,6b}$ = 12.4 Hz, H-6a), 3.86 (dd, 1H, $J_{5,6b}$ = 4.5 Hz, H-6b), 4.08 (dd, 1H, $J_{1,2}$ = 11.0 Hz, H-2), and 5.70 (d, 1H, H-1). ^{13}C NMR (CD_3OD + D_2O, 3:4, v/v) δ 10.4 (CH_2CH_3), 30.3 (CH_2CH_3), 42.5, 46.1 (NCH_3×2), 54.0 (C-2), 61.9 (C-6), 70.8 (C-4), 76.5 (C-3), 81.7 (C-5), 90.0 (C-1), 178.5 (C=O), and 194.7 (C=S). HR-FAB-MS: *m/z* 339.1053 $[M+H]^+$ (calcd for $C_{12}H_{23}O_5N_2S_2$:339.1049). 2-Butyramido-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (R^3): mp 171–172 °C (decomp.), $[\alpha]_D^{22}$ +49.6° (*c* 1.40, MeOH), IR(KBr) cm^{-1}: 3500–3150 (br OH, NH), 1643 (amide I), 1535 (amide II). ^{1}H NMR (CD_3OD + D_2O, 2.5:1, v/v) δ 0.93 (t, 3H, *J* = 7.4 Hz, CH_2CH_3), 1.62 (m, 2H, CH_2CH_3), 2.21 (m, 2H, $NCOCH_2$), 3.38, 3.53 (s, 6H, NCH_3×2), 3.46 (m, 1H, H-5), 3.48 (m, 1H, H-4), 3.62 (dd, 1H, $J_{2,3}$ = 9.2 Hz, $J_{3,4}$ = 8.5 Hz, H-3), 3.73 (dd, 1H, $J_{5,6a}$ = 4.6 Hz, $J_{6a,6b}$ = 12.4 Hz, H-6a), 3.86 (dd, 1H, $J_{5,6b}$ = 1.8 Hz, H-6b), 4.10 (dd, 1H, $J_{1,2}$ = 11.0 Hz, H-2), and 5.70 (d, 1H, H-1). ^{13}C NMR (CD_3OD + D_2O, 2.5:1, v/v) δ 13.9 (CH_2CH_3), 20.1 (CH_2CH_3), 39.0 ($NCOCH_2$), 42.3, 45.9 (NCH_3×2), 54.2 (C-2), 62.2 (C-6), 71.1 (C-4), 76.9 (C-3), 82.0 (C-5), 90.2 (C-1), 172.1 (C=O), and 195.1 (C=S). HR-FAB-MS: *m/z* 353.1209 $[M+H]^+$ (calcd for $C_{13}H_{25}O_5N_2S_2$: 353.1205). 2-Benzamido-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (R^4): $[\alpha]_D^{22}$ +85.1° (*c* 1.31, MeOH), IR(KBr) cm^{-1}: 3500–3200 (br OH, NH), 1641 (amide I), 1534 (amide II). ^{1}H NMR (D_2O) δ 3.25, 3.40 (s, 6H, NCH_3×2), 3.63 (dd, 1H, $J_{3,4}$ = 8.8 Hz, $J_{4,5}$ = 9.9 Hz, H-4), 3.69 (ddd, 1H, $J_{5,6a}$ = 5.1 Hz, $J_{5,6b}$ = 2.1 Hz, H-5), 3.82 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, H-6a), 3.93 (dd, 1H, $J_{2,3}$ = 9.9 Hz, H-3), 3.95 (dd, 1H, H-6b), 4.39 (dd, 1H, $J_{1,2}$ = 10.9 Hz, H-2), 5.96 (d, 1H, H-1), and 7.40–7.90 (m, 5H, aromatic protons). ^{13}C NMR (D_2O) δ 44.8, 48.3 (NCH_3×2), 56.8 (C-2), 63.6 (C-6), 72.5 (C-4), 78.0 (C-3), 83.4 (C-5), 91.8 (C-1), 130.0, 131.7, 135.3, 136.1 (aromatic carbons), 174.0 (C=O), and 195.7 (C=S). HR-FAB-MS: m/z 387.1041 $[M+H]^+$ (calcd for $C_{16}H_{23}O_5N_2S_2$:387.1049). 2-Deoxy-2-oleamido-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (R^5): $[\alpha]_D^{22}$ +40.9° (*c* 1.33, MeOH), IR (KBr) cm^{-1}: 3500–3200 (br OH, NH), 2924, 2853 (CH_2), 1643 (amide I), 1536 (amide II). ^{1}H NMR (CD_3OD) δ 0.90 (t, 3H, *J* = 6.8 Hz, CH_2CH_3), 1.23–2.25, 3.90 (m, 30H, methine and methylene protons of oleoyl group), 3.35, 3.50 (s, 6H, NCH_3×2), 3.38 (ddd, 1H, $J_{4,5}$ = 9.8 Hz, $J_{5,6a}$ = 5.1 Hz, $J_{5,6b}$ = 2.1 Hz, H-5), 3.43 (dd, 1H, $J_{3,4}$ = 8.6 Hz, H-4), 3.55 (dd, 1H, $J_{2,3}$ = 9.8 Hz, H-3), 3.68 (dd, 1H, $J_{6a,6b}$ = 12.0 Hz, H-6a), 3.82 (dd, 1H, H-6b), 4.09 (dd, 1H, $J_{1,2}$ = 11.0 Hz, H-2), and 5.67 (d, 1H, H-1). ^{13}C NMR (CD_3OD) δ 14.5 (CH_2CH_3), 23.7, 26.9, 27.5, 27.6, 30.1, 30.2, 30.3, 30.4, 30.6 (×2), 30.7, 33.0 (×2), 37.2, 39.6, 65.1 (methine and methylene carbons of oleoyl group), 41.9, 45.9 (NCH_3×2), 54.4 (C-2), 62.6 (C-6), 71.6 (C-4), 77.5 (C-3), 82.3 (C-5), 90.6 (C-1), 176.5 (C=O), and 195.4 (C=S). MS: *m/z* 569 $[M+Na]^+$.
17. 2-Amino derivative of OCT313 was synthesized by de-*O*-acetylation of 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate hydrochloride with an anion exchange resin DOWEX 1-X4 (OH^-) in methanol in 60.2% yields, which was obtained by the reaction of 2-*N*-anisilidene-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl bromide[13] and sodium *N,N*-dimethyldithiocarbamate in acetone, followed by deanisilidenation with 5 M hydrochloric acid. 2-Amido-2-deoxy-β-D-glucopyranosyl *N,N*-dimethyldithiocarbamate (R^6): mp 161–162 (decomp.), $[\alpha]_D^{23}$ −77.4° (*c* 1.04, H_2O), IR(KBr) cm^{-1}: 3500–3100 (br OH, NH). ^{1}H NMR (D_2O) δ 3.02 (dd, 1H, $J_{1,2}$ = 10.7 Hz, $J_{2,3}$ = 9.0 Hz, H-2), 3.44, 3.55 (s, 6H, NCH_3×2), 3.47 (dd, 1H, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 9.5 Hz, H-4), 3.50 (dd, 1H, H-3), 3.59 (ddd, 1H, $J_{5,6a}$ = 5.3 Hz, $J_{5,6b}$ = 2.2 Hz, H-5), 3.74 (dd, 1H, $J_{6a,6b}$ = 12.5 Hz, H-6a), 3.89 (dd, 1H, H-6b), and 5.62 (d, 1H, H-1). ^{13}C NMR (D_2O) δ 44.9, 48.4 (NCH_3×2), 57.3 (C-2), 63.5 (C-6), 72.2 (C-4), 80.4 (C-3), 83.3 (C-5), 93.2 (C-1), and 195.7 (C=S). HR-FAB-MS: *m/z* 283.0776 $[M+H]^+$ (calcd for $C_9H_{19}O_4N_2S_2$: 283.0786).

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Tuberculosis infection among homeless persons and caregivers in a high-tuberculosis-prevalence area in Japan: a cross-sectional study

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Abstract

Background: Tuberculosis (TB) is a major public health problem. The Airin district of Osaka City has a large population of homeless persons and caregivers and is estimated to be the largest TB-endemic area in the intermediate-prevalence country, Japan. However, there have been few studies of homeless persons and caregivers. The objective of this study is to detect active TB and to assess the prevalence and risk factors for latent TB infection among homeless persons and caregivers.

Methods: We conducted a cross-sectional study for screening TB infection (active and latent TB infections) using questionnaire, chest X-ray (CXR), newly available assay for latent TB infection (QuantiFERON-TB Gold In-Tube; QFT) and clinical evaluation by physicians at the Osaka Socio-Medical Center Hospital between July 2007 and March 2008. Homeless persons and caregivers, aged 30-74 years old, who had not received CXR examination within one year, were recruited. As for risk factors of latent TB infection, the odds ratios (OR) and 95% confidence intervals (95% CI) for QFT-positivity were calculated using logistic regression model.

Results: Complete responses were available from 436 individuals (263 homeless persons and 173 caregivers). Four active TB cases (1.5%) among homeless persons were found, while there were no cases among caregivers. Out of these four, three had positive QFT results. One hundred and thirty-three (50.6%) homeless persons and 42 (24.3%) caregivers had positive QFT results. In multivariate analysis, QFT-positivity was independently associated with a long time spent in the Airin district: ≥10 years versus <10 years for homeless (OR = 2.53; 95% CI, 1.39-4.61) and for caregivers (OR = 2.32; 95% CI, 1.05-5.13), and the past exposure to TB patients for caregivers (OR = 3.21; 95% CI, 1.30-7.91) but not for homeless persons (OR = 1.51; 95% CI, 0.71-3.21).

Conclusions: Although no active TB was found for caregivers, one-quarter of them had latent TB infection. In addition to homeless persons, caregivers need examinations for latent TB infection as well as active TB and careful follow-up, especially when they have spent a long time in a TB-endemic area and/or have been exposed to TB patients.

Background

Globally, there were an estimated 9.27 million cases of tuberculosis (TB) in 2007, with the larger number of cases of latent TB infection [1]. The Airin district of Osaka City is known as an urban area with a dense population of day-laborers and homeless people in Japan, an intermediate-prevalence country [2]. The prevalence of active TB in the Airin district was approximately 1,000 per 100,000 in the early 2000s, which was the highest in Japan and similar to that in developing countries [1,3,4]. In the Airin district, there are estimated to be 15,000 to 20,000 homeless persons with no medical insurance, accounting for 80% of visitors to a free or low-cost hospital, i.e. Osaka socio-medical center

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hospital [3]. TB screening programs have been carried out at Airin Health Office since 1990 and at the Osaka socio-medical center hospital since 2005. Mobile screening for TB was carried out monthly between 1973 and 2005, and weekly since 2006, by the Osaka City government on the streets of the Airin district [5]. The directly observed therapy short course (DOTS) program for homeless persons has been carried out since 1999. To date, several communities (i.e., NGOs, hospitals and facilities) for accommodation, health and welfare have been organized for homeless persons, following the Stop TB Strategy [5,6]. The prevalence of active TB in Osaka City decreased by 50% from 2000 to 2008. However, there are still estimated to be many unknown active TB cases in the Airin district [5].

Previous studies have identified risk factors for TB, such as immigrants, HIV, poverty, incarceration, smoking and alcohol use [7-10]. Homelessness-related TB remains a widespread problem [7]. An anti-TB strategy targeting homeless people, empowering caregivers and communities, and promoting research has been recommended [6,7]. However, studies on TB infection of homeless persons and their caregivers are scarce [11-13].

The commercially available blood test, QuantiFERON-TB Gold In-Tube (QFT; Cellestis Limited, Carnegie, Australia), is an interferon-gamma release assay (IGRA) in response to *M. tuberculosis*-specific antigens [14], and has been validated [15]. The QFT has excellent specificity and gives us valuable information of latent TB infection, even for a Japanese Bacillus Calmette-Guérin (BCG)-vaccinated population, whereas the accuracy of the tuberculin skin test (TST) is hampered by poor specificity due to the widespread use of BCG vaccination and re-vaccination in Japan [16].

The principal mechanism for the Stop TB Strategy is the detection and treatment of patients with TB [1]. The objectives of this study were to detect active TB cases and to assess the prevalence and risk factors of latent TB infection among homeless persons and their caregivers in a TB-endemic area in an intermediate-prevalence country.

Methods

Participants and Measurements

We conducted a cross-sectional study of homeless persons and their caregivers in the Airin district. Homeless persons were defined as persons who had had no permanent residence for more than one month. Caregivers were defined as persons who worked and supported homeless persons with regard to job arrangement, medical care, food supply, accommodation and clothing in the Airin district. Caregivers who belonged to five NGOs (mainly associated with a homeless shelter, job assistance, anti-alcoholism action, a soup-run and DOTS), as well as staff at the Osaka socio-medical center hospital and two clinics (nurses, social workers, dietitians, counselors and physicians) were enrolled in this study. Through our network of five NGOs, Osaka socio-medical center hospital and two local clinics by the use of posters, handouts and personal communication through a study recruiter, homeless persons and caregivers aged 30-74 years who had not received chest X-ray (CXR) examination within one year were recruited for tuberculosis screening at Osaka socio-medical center hospital between July 2007 and March 2008.

CXR, QFT, questionnaire and clinical evaluation by physicians were performed. If a participant had the symptom of sputum, smear testing and culture of sputum were performed for active TB diagnosis. A physician's interview using a standardized questionnaire covered the following data: age, sex, past history of TB, use of immunosuppressive drugs, past exposure to patients diagnosed with tuberculosis, current smoking and drinking status (more than 10 g of ethanol almost every day, yes or no), present symptoms of cough and/or sputum, general fatigue, elevated body temperature (≥37.0°C) and years spent living and/or working in the Airin district. Past exposure to tuberculosis patients was defined as self-reported exposure through living and/or working with TB patients in a shared space before the patients had been diagnosed with active TB.

CXR findings were categorized into two groups, normal and abnormal, based on our standard method [4]. Further classification was not performed since TB shadows vary and could take any kind of shape [17]. Quality control was performed by double-checks on each radiograph by another TB specialist. Active TB case was defined as an individual with symptoms compatible with TB plus detection of nucleic acid from mycobacterium tuberculosis complex from a clinical specimen, or as a patient with tuberculosis clinically suspected by an expert physician plus a response to anti-tuberculosis treatment [18].

QFT was performed and interpreted according to the manufacturer's instructions, with an interferon-γ response for the tuberculosis antigen tube minus Nil of ≥0.35 IU/ml defined as a positive result. QFT results were considered indeterminate if the subject did not respond to the mitogen-positive control tube with at least 0.5 IU/ml of interferon-γ [19,20].

Written informed consent was provided by each participant and each received 500 yen (about five US dollars) as an incentive to participate. The ethical committees of Osaka socio-medical center hospital and Osaka University approved this study.

Statistical Analysis

The proportions of basic characteristics and TB-related findings were descriptively shown among homeless persons and caregivers. The prevalence and 95% confidence

intervals (95% CI) for active TB were calculated using method based on the F-distribution.

The odds ratios (OR) and 95% CI for QFT-positivity were calculated using logistic regression model. We assessed how the positive QFT prevalence varied according to the potential factors such as length of time spent living and/or working in the Airin district and past exposure to TB patients, using the dichotomized categories to avoid statistical instability derived from small sample size. We investigated the multivariate model among homeless persons and caregivers, who did not have active TB disease or indeterminate QFT result.

Because of the failure in convergence, we removed the variables that had <5% cases with exposure or non-exposure category from the age-adjusted and multivariate logistic model [21]. Thereby, female and elevated body temperature for homeless, cough and/or sputum, elevated body temperature, past history of TB and abnormal chest X-ray finding for caregivers were excluded from the analyses.

Probability values for statistical tests were two-tailed and $p < 0.05$ was regarded as statistically significant. The SAS statistical software package (version 9.1; SAS Institute Inc., Cary, NC, USA) was used for all analyses.

Results

A total of 448 persons were enrolled in the study. However, of these 448 participants, four homeless persons refused to give a blood sample, and eight homeless persons left the waiting room before having a CXR and/or completing a questionnaire. Thus, complete results of CXR, QFT and questionnaire were available for 436 participants (263 homeless persons and 173 caregivers).

Table 1 shows the basic characteristics and TB-related findings for homeless persons and caregivers. The homeless persons included only two women and exhibited higher values for almost all variables of interest than caregivers. Four active TB cases were found among the homeless persons: the prevalence of 1.52% (95% CI, 0.42-3.85), while there were no cases among caregivers. One-half of the homeless persons (50.6%) and one-quarter of the caregivers (24.3%) had a positive QFT result.

Table 2 shows the characteristics of active TB cases. Out of the four active TB cases, all four subjects were homeless, male, more than 60 years of age, had abnormal CXR findings, had lived or worked for more than 9 years (mean 13 years) in the Airin district and had symptoms of cough and/or sputum. Three subjects had positive QFT results. None of them had acknowledged past exposure to TB patients.

Table 3 shows age-adjusted and multivariate OR (95% CI) of QFT-positivity according to potential risk factors among homeless persons and caregivers, who did not have active TB disease or indeterminate QFT result. Both homeless persons and caregivers who had spent more than ten years in the Airin district had significantly higher positive QFT result than those who had spent less than ten years in both age-adjusted and multivariate models. When we used a cutoff point of half of the period, namely, five years, homeless persons who had spent more than five years had significantly higher positive QFT result (multivariate odds ratio, 2.93; 95% CI, 1.43-6.01) but caregivers did not (multivariate odds ratio, 1.93; 95% CI, 0.84-4.45). Current drinker was a significant predictive factor for QFT-positivity among homeless persons, but not among caregivers in both age-adjusted and multivariate models. The caregivers who had acknowledged past exposure to TB patients had significantly higher QFT-positivity than those who did not in both age-adjusted and multivariate models, while the homeless persons did not in the multivariate model.

Past history of TB and abnormal chest X-ray finding were not associated significantly with positive QFT result among homeless persons in the multivariate model, and the analysis was not carried out for caregivers owing to the small number with a past history or abnormal chest X-ray finding.

Discussion

Active TB prevalence

We showed that homeless people in the Airin district remain at high risk for active TB, and that the cross-sectional estimate of the prevalence of active TB was a rate of 1.52% (95% CI, 0.42-3.85) among homeless people in the Airin district, Osaka, Japan. A similar high prevalence of active TB was reported among homeless day-laborers (2.2%) in the same district from a previous study in 2003-2005 [4]. According to annual TB patients' registry database, the prevalence of active TB among residents in the Airin district was 653 per 100,000 (0.65%) in 2007, and the homeless people accounted for 79.3% of TB patients in that district [22]. These estimates of active TB prevalence were markedly higher than those of the total Japanese population and the Osaka City population in 2007, namely, 19 per 100,000 (0.019%) and 53 per 100,000 (0.053%), respectively [1,22].

High prevalence of active TB among the homeless population was reported from other countries. In New York City, McAdam et al. reported the high active TB prevalence of 1,502 per 100,000 among homeless persons in 1992 [23]. In London, Story et al. showed the high prevalence of 788 per 100,000 among homeless people in 2003 [24]. TB is concentrated in the homeless population in developed countries and TB transmission may occur from homeless people to other populations [25].

Table 1 Basic characteristics and TB-related findings of the study participants

		Homeless persons (n = 263)	Caregivers (n = 173)
Basic characteristics			
Sex, female	n (%)	2 (0.8)	94 (54.3)
Age, years	mean median (range)	58.0 59 (30-74)	48.2 48 (30-71)
30-49	n (%)	40 (15.2)	97 (56.1)
50-59	n (%)	108 (41.1)	52 (30.1)
60-74	n (%)	115 (43.7)	24 (13.9)
Current smoker	n (%)	189 (73.0)	63 (37.1)
Current drinker	n (%)	134 (51.9)	54 (32.0)
Length of time spent living and/or working in the Airin district, years	mean median (range)	13.5 10 (0.1-58)	10.3 5 (0.1-64)
<5 years	n (%)	63 (25.9)	73 (43.2)
5-10 years	n (%)	46 (18.9)	33 (19.5)
≥10 years	n (%)	134 (55.1)	63 (37.3)
TB-related findings			
Past history of TB	n (%)	33 (12.6)	6 (3.5)
Past exposure to TB patients	n (%)	50 (19.8)	32 (18.7)
Cough and/or sputum	n (%)	64 (24.6)	6 (3.5)
General fatigue	n (%)	51 (19.6)	9 (5.2)
Elevated body temperature	n (%)	9 (3.5)	1 (0.6)
Abnormal chest X-ray finding	n (%)	68 (25.9)	7 (4.1)
Active TB case	n (%)	4 (1.5)	0 (0.0)
Positive QFT result	n (%)	133 (50.6)	42 (24.3)
Indeterminate QFT result	n (%)	4 (1.5)	1 (0.6)

Abbreviations: TB, tuberculosis; QFT, QuantiFERON-TB Gold In-Tube.

The number of missing values was 20 for length of time spent in the Airin district, 10 for past exposure to TB patients, 4 for smoking, 5 for drinking and 3 for cough and/or sputum, general fatigue and elevated body temperature among homeless persons, and 4 for length of time spent in the Airin district, 2 for past exposure to TB patients, 3 for smoking and 4 for drinking among caregivers.

None of the participants had a history of extra-pulmonary TB or used immunosuppressive agents.

The trends for active TB prevalence among homeless people varied considerably by location. In New York City, the prevalence had a substantial decline, that is, 1,502 per 100,000 in 1992 to 171 per 100,000 in 2004 [23]. In the Airin district, where approximately 80% of residents were estimated to be homeless people, the prevalence declined substantially from 1400 per 100,000 in 2000 to 680 in 2005, and declined further to a small extent to 653 per 100,000 in 2007 [22].

The substantial decline in the active TB prevalence in New York City and the moderate decline in the Airin district among homeless people were attributable

Table 2 Characteristics of active TB cases

	Case 1	Case 2	Case 3	Case 4	Total*
Homeless or caregivers	Homeless	Homeless	Homeless	Homeless	4
Sex	Male	Male	Male	Male	4
Age	62	63	66	66	Mean 64.3
Current smoker	-	+	+	-	2
Current drinker	-	+	-	+	2
Length of time spent living and/or working in the Airin district, years	10	9	20	13	Mean 13.0
Past history of TB	-	+	-	-	1
Past exposure to TB patients	-	-	-	-	0
Cough and/or sputum	+	+	+	+	4
General fatigue	+	-	+	+	3
Elevated body temperature	-	-	-	-	0
Abnormal chest X-ray finding	+	+	+	+	4
QFT-positivity	-	+	+	+	3

Abbreviations: TB, tuberculosis; QFT, QuantiFERON-TB Gold In-Tube.

*The number of positive for each factor or mean value for continuous variables among four active TB cases.

Table 3 Age-adjusted and multivariate odds ratios (OR) and 95% confidence intervals (95% CI) of QFT-positivity according to potential risk factors among homeless persons and caregivers, after excluding the subjects with active TB disease or indeterminate QFT results

		Homeless persons (n = 255)				Caregivers (n = 172)			
		No. of subjects	No.(%) of QFT-positivity	Age-adjusted OR (95% CI)	MultivariateOR (95% CI)†	No. of subjects	No.(%) of QFT-positivity	Age-adjusted OR (95% CI)	MultivariateOR (95% CI)†
Sex	Male	253	130 (51.4)	NA	NA	79	20 (25.3)	1.0	1.0
	Female	2	0 (0.0)	NA	NA	93	22 (23.7)	0.95 (0.46-1.93)	0.91 (0.39-2.11)
Current smoker	No	66	33 (50.0)	1.0	1.0	106	27 (25.5)	1.0	1.0
	Yes	185	93 (50.3)	1.19 (0.66-2.14)	1.23 (0.63-2.40)	63	13 (20.6)	0.81 (0.38-1.75)	0.72 (0.29-1.74)
Current drinker	No	121	56 (46.3)	1.0	1.0	114	26 (22.8)	1.0	1.0
	Yes	129	70 (54.3)	1.74 (1.02-2.96)*	1.84 (1.01-3.37)*	54	13 (24.1)	1.23 (0.56-2.69)	1.15 (0.48-2.74)
Length of time spent living and/or working in the Airin district, year	<10 years	107	38 (35.5)	1.0	1.0	105	18 (17.1)	1.0	1.0
	≥10 years	128	78 (60.9)	2.52 (1.46-4.35)*	2.53 (1.39-4.61)*	63	24 (38.1)	2.60 (1.24-5.42)*	2.32 (1.05-5.13)*
Past exposure to TB patients	No	196	96 (49.0)	1.0	1.0	138	27 (19.6)	1.0	1.0
	Yes	50	31 (62.0)	2.05 (1.05-3.99)*	1.51 (0.71-3.21)	32	14 (43.8)	3.63 (1.55-8.47)*	3.21 (1.30-7.91)*
Cough and/or sputum	No	194	100 (51.6)	1.0	1.0	166	42 (25.3)	NA	NA
	Yes	59	29 (49.2)	0.91 (0.50-1.67)	0.64 (0.32-1.32)	6	0 (0.0)	NA	NA
General fatigue	No	207	102 (49.3)	1.0	1.0	163	40 (24.5)	1.0	1.0
	Yes	46	27 (58.7)	1.66 (0.84-3.29)	1.47 (0.66-3.28)	9	2 (22.2)	0.96 (0.19-4.93)	1.06 (0.19-5.96)
Elevated body temperature	No	244	124 (50.8)	NA	NA	171	42 (24.6)	NA	NA
	Yes	9	5 (55.6)	NA	NA	1	0 (0.0)	NA	NA
Past history of TB	No	225	109 (48.4)	1.0	1.0	166	37 (22.3)	NA	NA
	Yes	30	21 (70.0)	2.23 (0.95-5.21)	1.51 (0.43-5.31)	6	5 (83.3)	NA	NA
Abnormal chest X-ray finding	No	193	86 (44.6)	1.0	1.0	165	37 (22.4)	NA	NA
	Yes	62	44 (71.0)	2.62 (1.39-4.93)*	1.90 (0.78-4.60)	7	5 (71.4)	NA	NA

*$p < 0.05$.

Abbreviations: TB, tuberculosis; NA, not applicable.

†Multivariate-adjusted for the listed factors and age.

to intensive population-based TB screening and DOTS [5,26].

Prevalence of latent TB infection

We found high prevalence of QFT-positivity for homeless persons (50.6%) and for caregivers (24.3%) at the ages of 30-74 years. When we restricted the sample to those of ages 40-69 years, the respective proportions were 50.4% for homeless persons and 30.8% for caregivers. These prevalences were far higher than that of the general Japanese population aged 40-69 years, which is estimated to be 7.1% [27].

Latent TB infection among vulnerable persons including homeless people

Garfein et al. investigated latent TB infection among 280 homeless persons in a Mexican city with the highest TB prevalence using an IGRA and found the prevalence of QFT-positivity was 51.8% [28], which was nearly equal to the prevalence in our study. In addition to homeless people, high-risk individuals for latent TB infection can be detected by the IGRA. For example, the prevalence of IGRA-positivity was reported to be 29.8% among immigrants, mostly from Latin America, in Italy [29], 33.6% among drug users in Houston, USA [30], and 53.9% among immigrants with close contact to sputum smear-positive TB patients in Netherlands [31].

Latent TB infection among caregivers

A study in Italy showed that the prevalence of latent TB infection (positive QFT) was 55.5% among caregivers working at a homeless shelter [11], which showed higher latent TB prevalence than the caregivers in our study. However, compared with healthcare workers for latent TB infection, the caregivers of our study had higher prevalence of latent TB infection. The prevalence of latent TB infection among healthcare workers in low- to intermediate-prevalence countries including Japan ranged from 1% to 19% [15,32-35], which was much lower than that of the caregivers in our study. Mirtskhulava et al. reported an extremely high prevalence of latent TB infection (60.0%) among healthcare workers, probably because they had frequent contact with TB patients and also high prevalence of TB in the community [36].

Putative risk factors for latent TB infection

Another aim of our study was to determine putative risk factors for latent TB infection. QFT-positivity was associated with past exposure to TB patients among caregivers, but not among homeless people. Caregivers usually know when and how they have been in contact with people with TB, whereas homeless persons often ignored or did not notice this [37]. Homeless people who drank almost every day had higher QFT-positivity than those who did not. Habitual drinkers may be more likely to have contact with other drinkers and had a higher risk of being infected [38]. The past history of TB was not significantly associated with a positive QFT result among homeless persons, which might be related to the waning of immune responses in the time course of TB infection [27]. The duration of living and/or working in Airin district was associated with QFT-positivity among both homeless people and caregivers, but this was the case only for ≥ 5 years among homeless people.

The risk of QFT-positivity was found to increase with increasing length of time spent in the Airin district, independently of acknowledgement of exposure to TB patients. Even caregivers without known exposure to TB patients in this study had approximately twofold higher QFT-positivity (19.6%, Table 3) than healthcare workers in Japan (9.9%) [16], indicating that they may be at high risk for TB infection. As mentioned above, caregivers at a homeless shelter had high latent TB prevalence even in a low-prevalence country [11]. Thus, anti-TB measures for caregivers should be strengthened to ensure their safety.

Limitations

This study has several potential limitations. The setting and selection of homeless persons in our study may limit the ability to generalize our results to the entire Airin district. Our sample of homeless persons may be at high risk to have been in contact with TB patients. The information on homelessness, past history of TB, past exposure to TB patients and length of time spent living and/or working in the Airin district was self-reported. Homeless people may be less likely to recall such information accurately because they are less health-conscious. The cross-sectional nature of the data limits the degree to which we can assign causality, especially with respect to temporality. However, it might be plausible that the exposure, such as past exposure to TB patients and length of time spent in the Airin district, may precede TB infection or disease. Information regarding TB-related factors such as HIV infection, drug abuse and history of incarceration was not included in the data collection. However, it is well known that TB/HIV co-infection is quite low in Japan [39]. In the present study, TST was not carried out because we wanted to avoid the refusal of study participation by homeless persons, and there is poor agreement between TST and QFT results caused by the effect of BCG vaccination in Japan [16].

Conclusions

We found that the prevalence of latent TB infection was approximately 50% for homeless people and 25% for

caregivers, and a long duration spent by both groups in the Airin district in Osaka, Japan, was associated with latent TB infection. Although no active TB was found for caregivers, one-quarter of them had latent TB infection. In addition to homeless persons, caregivers need examinations for latent TB infection as well as active TB and careful follow-up, especially when they have spent a long time in a high TB prevalence area and/or have been exposed to TB patients.

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Authors' contributions

T. Takatorige, YH, NN, SH, AS, KF, HY and T. Takashima participated in the planning of the study. T. Takatorige and T. Takashima coordinated the study and took overall responsibility for the delivery of the work. T. Tabuchi, YH, KF and HY had responsibility for data collection. T. Tabuchi conducted the analysis, with statistical support from HI, YT, TN and TM. T. Tabuchi, HI and T. Takashima participated in writing the paper. All authors participated in the interpretation of the study and read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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