the inhibition of both the caveola-dependent endocytosis and caveola-independent lipid-raft-dependent endocytosis (18, 32). HepG2 cells were treated with various concentrations of MBCD for 30 min and thereafter infected with the pseudoviruses in the absence of MBCD to avoid any potential effect of MBCD on the viral envelopes (Fig. 1B). MBCD treatment inhibited the susceptibility of HepG2 cells to A-MLV(HIV), to 18% of the control level at 2500 μM MBCD, whereas only a modest reduction in the cells' susceptibility to the SARS-CoV-(HIV) and VSV(HIV) was seen, to 66 and 53% of the control, respectively (Fig. 1B).

We next used SARS-CoV (Vietnam/NB-04/2003). HepG2 cells were treated or untreated with indicated concentrations of chlorpromazine for 1 h and then infected with SARS-CoV for 24 h. Infection efficiency of SARS-CoV was determined by RT-PCR. It was significantly inhibited by chlorpromazine treatment (Fig. 1C). Collectively, these results suggest that SARS-CoV entry into HepG2 cells is mostly mediated by the clathrin-dependent pathway, although some SARS-CoV pseudovirus entry appears to be dependent on caveolae and/or lipid rafts.

Effect of CHC depletion on SARS-CoV entry. To examine whether clathrin-mediated endocytosis is required for SARS-CoV(HIV) entry, we used siRNA-mediated gene silencing against the major component of the clathrin triskelion, CHC. In addition, under this clathrin knockdown condition, we simultaneously inhibited the clathrin-independent pathways with MBCD. This method was designed to determine whether the pseudoviruses entered the cells via the clathrin-dependent or -independent pathways, or both.

Transfection of a specific siRNA for CHC reduced the CHC protein expression to less than 10% of that in the control siRNA-transfected HepG2 cells, and we observed no effect of MBCD treatment on the CHC knockdown (Fig. 2A). CHC-depleted MBCD-treated cells, CHC-depleted mock-treated cells, and control cells were infected with the SARS-CoV(HIV), VSV(HIV), and A-MLV(HIV). CHC depletion reduced the SARS-CoV-(HIV) infectivity to 38% and the infectivity of VSV(HIV) to 42% of that in the control cells. Cholesterol depletion by MBCD in the CHC knockdown cells reduced the SARS-CoV(HIV) infectivity to 28% and the infectivity of VSV(HIV) to 36% of their infectivity in the control cells (Fig. 2B). On the other hand, there was little effect on the A-MLV(HIV) infectivity in cells expressing the siRNA. However, MBCD treatment markedly reduced the A-MLV(HIV) infectivity to 41% (Fig. 2B). The *t* test revealed the statistical significances in combinations between MBCD-treated and -untreated CHC knockdown cells infected with the pseudoviruses.

We also examined the effect of clathrin knockdown on SARS-CoV (Vietnam/NB-04/2003) infection to HepG2 cells under a similar condition to the pseudoviruses. CHC depletion reduced the SARS-CoV infectivity to 65% of that in the control cells at 24 h postinfection. Cholesterol depletion by MBCD in the CHC-knockdown cell slightly inhibited to this effect (Fig. 2C). These results support the evidence described above that the clathrin-mediated pathway of endocytosis is required for an efficient SARS-CoV entry into HepG2 cells.

Receptor activities of ACE2 mutants for SARS-CoV infection. To further investigate the clathrin-dependent endocytosis of SARS-CoV, we sought to determine whether the cytoplasmic domain of ACE2 is required for interaction with AP2/

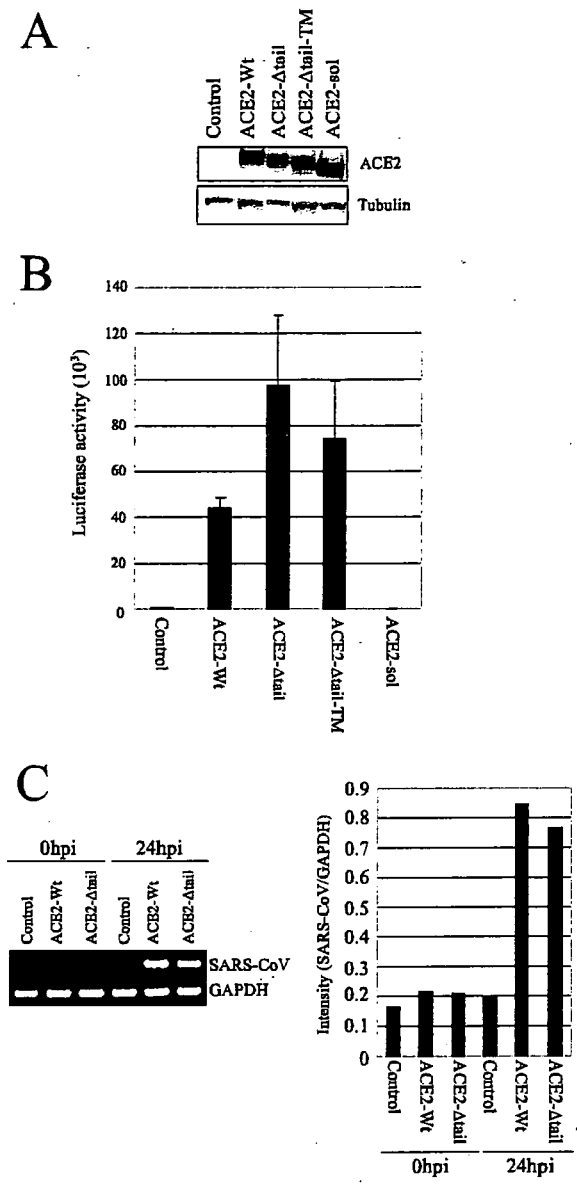

FIG. 3. Receptor activity of ACE2 mutants for pseudoviruses and SARS-CoV infection. (A) COS7 cells were transiently transfected with ACE2-wt, ACE2-Δtail, ACE2-Δtail-TM, ACE2-sol or control plasmids, and after 48 h of transfection their lysates were tested by immunoblotting with anti-ACE2 or anti-tubulin monoclonal antibody. (B and C) The transfected cells were infected with SARS-CoV(HIV) (B) or SARS-CoV (Vietnam/NB-04/2003) (C). Their luciferase activities were measured in triplicate, and their expressions of viral RNA were measured by RT-PCR.

clathrin complexes. We prepared an ACE2 mutant (ACE2-Δtail) that lacks the cytoplasmic domain by introduction of the stop codon at the end of the transmembrane domain of ACE2. The virus receptor activity of ACE2 mutant was examined with COS7 cells because COS7 cells are negative for ACE2 expression detected by RT-PCR and immunoblotting but positive for caveolin-1 (data not shown) (34). COS7 cells transfected with ACE2-Δtail were infected with SARS-CoV(HIV) equally well to the cells transfected with the wild-type ACE2 (Fig. 3A and

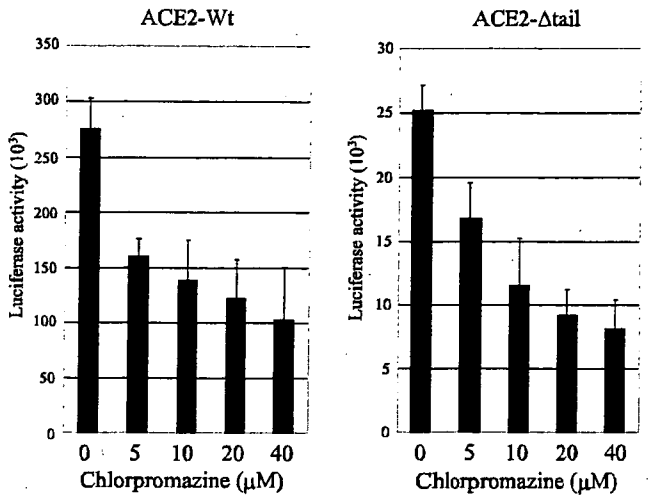

FIG. 4. Effects of chlorpromazine on pseudovirus infection to cells expressing ACE2-Δtail. COS7 cells were transiently transfected with ACE2-wt or ACE2-Δtail and then treated with the indicated amounts of chlorpromazine. Subsequently, the cells were infected with SARS-CoV(HIV). Their luciferase activities were measured in triplicate.

B). We further examined the receptor activity of ACE2-Δtail-TM, which lacks the cytoplasmic domain and replaces the transmembrane domain with that derived from EGFR. ACE2-Δtail-TM also showed a receptor activity for SARS-CoV(HIV) (Fig. 3B). We also confirmed that ACE2-sol, a soluble form of the ACE2 extracellular domain, has no receptor activity for SARS-CoV(HIV). Next, we used SARS-CoV (Vietnam/NB-04/2003). COS7 cells transfected with ACE2-Δtail were also infected with SARS-CoV equally well to the cells transfected with the wild-type ACE2 at 24 h postinfection (Fig. 3C). These results suggest that the cytoplasmic domain of ACE2 is not essential for its receptor activity and that there is no specificity of the transmembrane domain for its receptor activity.

We next confirmed that the ACE2-Δtail-mediated infection of SARS-CoV(HIV) is also clathrin dependent. COS7 cells transfected with the wild-type ACE2 or ACE2-Δtail were pretreated with chlorpromazine and infected with SARS-CoV-(HIV). The chlorpromazine treatment induced suppression of SARS-CoV(HIV) infection to COS7 cells expressing ACE2-Δtail, as well as the wild-type ACE2 (Fig. 4).

SARS-CoV(HIV) is transported into EEA1-positive early endosomes. Accumulating evidence suggests that cell surface molecules internalized by the clathrin-dependent pathway are transferred into early endosomes. We used confocal microscopy to examine whether ACE2 is internalized in early endosomes upon SARS-CoV(HIV) binding. After a 3 h period of serum starvation, HepG2 cells expressed ACE2 predominantly on the cell surface (Fig. 5, upper panels). The cells were then infected with SARS-CoV(HIV) concentrated 10-fold by ultracentrifuge. By 10 min after the infection, the ACE2 localization had changed dramatically, from the cell surface to EEA1-positive early endosomes (Fig. 5, lower panels). We also confirmed that the SARS-CoV(HIV) entry was affected by acidification inhibitors such as ammonium chloride (NH_4Cl) and chloroquine (data not shown). Furthermore, we examined the effect of ammonium chloride on SARS-CoV(HIV) infection into COS7 cells expressing ACE2-

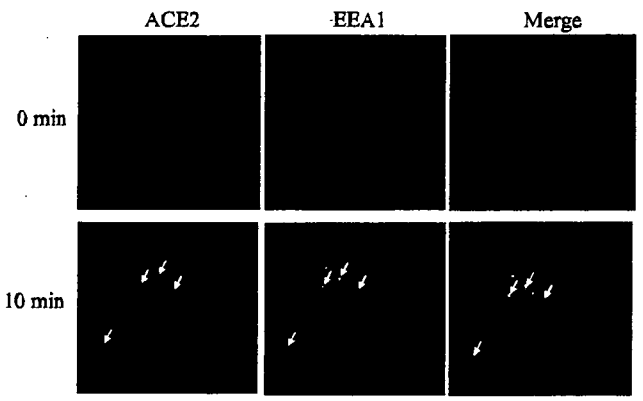

FIG. 5. Immunohistochemical localization of ACE2 after pseudovirus infection. HepG2 cells were cultured in the FCS-free medium to induce ACE2 on cell surfaces and incubated with concentrated SARS-CoV-(HIV) for 10 min at 37°C. They were then stained for ACE2 and EEA1 by immunofluorescence.

Δtail (Fig. 6). The ammonium chloride treatment induced inhibition of SARS-CoV(HIV) infection in a manner similar to that for HepG2 cells. These results suggest that the binding of the SARS-CoV(HIV) to ACE2 induces rapid internalization of the ACE2/pseudovirus complex into EEA1-positive early endosomes, where a low pH condition is required for it to establish an infection and that the cytoplasmic tail of ACE2 is not required for the internalization of SARS-CoV(HIV) into endosomes.

DISCUSSION

Productive infection of target cells by animal viruses requires their access to highly specific entry pathways that allow

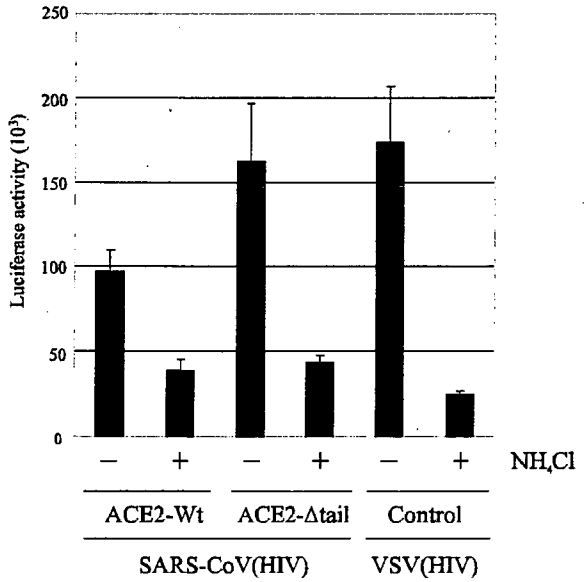

FIG. 6. Dependence on acidic environment for infection by the pseudoviruses. COS7 cells transiently transfected with ACE2-wt, ACE2-Δtail, or control plasmids were treated with 20 mM NH_4Cl and then infected with SARS-CoV(HIV) and VSV(HIV). Their infectivities were evaluated by measuring the luciferase activity. The experiment was performed in triplicate.

critical virion components to be introduced into the cytoplasm for subsequent processes, including uncoating, gene expression, and replication. The present study documented that the main pathway of SARS-CoV entry into host cells is dependent on clathrin. Chlorpromazine is a cationic amphiphilic agent that inhibits the formation of clathrin-coated pits (24). The use of chlorpromazine has established that a number of other viruses, including VSV (40) and influenza virus (36), use clathrin for entry into the host cell. We found that chlorpromazine inhibited the infection of HepG2 cells by SARS-CoV(HIV) more significantly than by VSV(HIV). We also confirmed the inhibitory effect of chlorpromazine on the SARS-CoV (Vietnam/NB-04/2003) infection to HepG2 cells. Moreover, HepG2 cells used here are unable to form caveolae because of no expression of caveolin-1 (data not shown). These results suggest that the SARS-CoV entry is mainly mediated by clathrin-coated pits. The specificity of the chlorpromazine effect was supported by the observation that the chlorpromazine treatment had little effect on the entry of the clathrin-independent A-MLV(HIV). To verify SARS-CoV's dependence on clathrin for host cell entry, we used CHC knockdown HepG2 cells as the target for SARS-CoV infection. The treatment of HepG2 cells with CHC siRNA induced significant suppression of the SARS-CoV(HIV) infection, as well as the SARS-CoV infection. Furthermore, we found that the ACE2 is colocalized with the CHC out of the lipid raft. Hence, although we cannot exclude a minor contribution of the clathrin-independent pathway to SARS-CoV(HIV) entry, the clathrin-dependent pathway is probably the main one used by SARS-CoV for host cell entry.

A previous report demonstrated that an ACE2 mutant partially deleted of the cytoplasmic domain sustains a receptor activity for SARS-CoV infection (11). Since we found that the ACE2-mediated infection of SARS-CoV is mainly clathrin dependent, we then definitively sought to determine whether the cytoplasmic domain of ACE2 is required for the clathrin-dependent pathway of SARS-CoV infection. We prepared an ACE2 mutant, ACE2-Δtail, with the cytoplasmic domain completely deleted, and demonstrated that the SARS-CoV infection via the ACE2-Δtail is mainly clathrin dependent because chlorpromazine inhibits the ACE2-Δtail-mediated entry of SARS-CoV(HIV) into COS7 cells. These results indicate that the cytoplasmic domain of the ACE2 is not essential for the clathrin-dependent entry of SARS-CoV, which suggests that there is a possible coreceptor for the ACE2, which interacts with the AP2/clathrin complex. The replacement of the transmembrane domain of ACE2 with that of EGFR showed no effect on the susceptibility of the cells to SARS-CoV. Since the ACE2 extracellular domain alone is unable to induce the receptor activity, the extracellular domain containing the transmembrane domain is indispensable for the receptor activity for SARS-CoV but the cytoplasmic tail of ACE2 is dispensable.

SARS-CoV infection was previously shown to be suppressed partially by treatment of the cholesterol-depleting reagent, MBCD (19). We also observed lower but significant levels of MBCD-mediated suppression of the SARS-CoV(HIV) and VSV(HIV) infection compared to the A-MLV(HIV) infection. The difference in MBCD's suppressive effects between A-MLV(HIV) and the other two pseudoviruses may be explained by the possibility that the SARS-CoV(HIV) entry might be partially mediated by a clathrin-independent pathway corresponding to the lipid raft-mediated pathway. Alternatively, in a previous report, MBCD partially inhibited the clathrin-mediated endocytosis of transferrin receptor and EGFR, as well as completely inhibiting their lipid raft- and/or caveola-mediated endocytosis (14, 33), suggesting that MBCD might have some unexpected effect on the clathrin-mediated pathway for the SARS-CoV infection. To address these possibilities, we investigated the additive or synergistic effect of MBCD with CHC-siRNA on the SARS-CoV infection to HepG2 cells. MBCD induced a weak but significant additive suppression on CHC-siRNA-treated HepG2 cell infection by SARS-CoV, suggesting a minor clathrin-independent entry pathway for SARS-CoV.

The SARS-CoV S protein is cleaved into S1 and S2 proteins by an acidic protease, cathepsin L, in endosomes, which is essential for fusion between the viral envelope and the endosome vesicular membrane (1, 13, 37). We demonstrated here the translocation of ACE2 to the EEA1-positive endosomes upon SARS-CoV(HIV) infection and showed the suppressive effects of acidification inhibitors, NH_4Cl and chloroquine, on SARS-CoV(HIV) infection to HepG2 cells. Furthermore, the SARS-CoV(HIV) infection to COS7 cells expressing ACE2-Δtail was significantly suppressed by NH_4Cl treatment, suggesting that SARS-CoV infection via ACE2-Δtail is not different from that via the wild-type ACE2.

Based on the present study, we propose a model for SARS-CoV's internalization by target cells. SARS-CoV attaches the cell surface through an interaction between the envelope spike glycoprotein and its receptor, ACE2. Clathrin-coated pits are then formed by interactions between the ACE2/virus complex and the AP2/clathrin complex via a possible coreceptor in a non-lipid-raft portion of the plasma membrane. The ACE2/virus complex is then translocated to endosomes, where the virus is uncoated by the help of endosomal acid protease, such as cathepsin L (13, 37).

Among the *Coronaviridae*, human coronavirus 229E (HCoV-229E) utilizes aminopeptidase N (CD13) as its receptor, which is localized in the lipid rafts, and HCoV-229E infection is inhibited by the treatment of target cells with MBCD and the transfection of an siRNA specific for caveolin-1, suggesting that HCoV-229E utilizes the caveola-mediated endocytosis pathway for its entry into target cells (26). Since CD13 is a common viral receptor for other group 1 coronaviruses, such as porcine transmissible gastroenteritis virus, porcine epidemic diarrhea virus, feline infectious peritonitis virus, and canine coronavirus, the caveola-mediated endocytosis pathway seems to be conserved among them. In contrast, murine hepatitis virus, which is a group 2 coronavirus, was previously shown to utilize the lipid raft-mediated endocytosis pathway (5). Hence, SARS-CoV, which is also classified into the group 2 coronavirus, seems to be unique among the *Coronaviridae* because it utilizes the clathrin-mediated pathway for its entry into HepG2 and COS7 cells.

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Anti-tuberculosis drug susceptibility testing of *Mycobacterium bovis* BCG Tokyo strain

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SUMMARY

SETTING: The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine is the only vaccine against tuberculosis (TB), owing to its valuable protective effects and low virulence. However, it can occasionally cause systemic infection in immunocompromised hosts. Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are known to be effective anti-tuberculosis drugs and are used for the treatment of BCG infections. Unfortunately, there are few studies of the susceptibility of BCG vaccine strains to these drugs.

OBJECTIVE: To measure the minimum inhibitory concentrations (MICs) of BCG Tokyo vaccine products for anti-tuberculosis drugs and assess vaccine safety in terms of drug susceptibility.

DESIGN: We measured the MIC for one seed and five product lots of BCG Tokyo strain for INH, RMP, SM and EMB using Middlebrook 7H11 agar plates.

RESULTS: The MIC results for INH were 0.06 and 0.125 μg/ml for the product and seed lots, respectively. The MIC results for RMP, SM and EMB were 0.25–0.5, 0.25 and 2–4 μg/ml, respectively.

CONCLUSION: Our results indicate that the BCG Tokyo strain was susceptible to the major anti-tuberculosis drugs and treatable even in cases of severe adverse events, including systemic infection.

KEY WORDS: BCG; minimum inhibitory concentration; drug susceptibility

TUBERCULOSIS (TB) is an infectious disease of international importance that remains a major life-threatening disease worldwide. It is estimated that approximately one third of the world's population is infected with *Mycobacterium tuberculosis*. Every year, approximately 9 million people develop active disease and 1.7 million die of TB.[1]

Bacille Calmette-Guérin (BCG) vaccines are safe, attenuated live bacteria and have been shown to have valuable protective effects against TB. The BCG Tokyo strain is recognised as a low virulence strain among all BCGs,[2] and is widely used in several countries as a vaccine strain. If used properly, it protects against the development of TB and the dissemination of TB bacilli. Few severe complications have been reported.[3] However, systemic BCG infection may occur frequently when it is administered to immunocompromised hosts with congenital or acquired immunodeficiency such as human immunodeficiency virus (HIV) infection.[4,5] BCG is contraindicated in symptomatic HIV diseases. When general BCG infection occurs, patients are treated empirically using anti-tuberculosis drugs because there is limited information about the drug susceptibility of BCG strains. It is therefore very important to evaluate the drug susceptibility of BCG Tokyo strain to ensure the safety of the vaccine.

Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are the first-line anti-tuberculosis drugs most commonly used in standard TB treatment regimens. These drugs are currently available even in developing countries. The present study aimed at measuring the minimum inhibitory concentrations (MICs) of these drugs against the BCG Tokyo strain to estimate the effect of clinical treatment in case of infection by the BCG Tokyo strain.

MATERIALS AND METHODS

BCG Tokyo strain

Five lots of vaccine product (number 1003 as 'Lot A', 1960 as 'Lot B', 1036 as 'Lot C', 1061 as 'Lot D', 1998 as 'Lot E') and one seed lot were provided by the Japan BCG Laboratory (Tokyo, Japan) and used in this study. These vaccines were produced by Japan BCG Laboratory for vaccination from the seed lot in 2004. The experiment was carried out in a type II-B

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Minimum inhibitory concentrations

The MICs were measured modifying the proportion method described in M24-A of the Clinical and Laboratory Standards Institute (CLSI, former National Committee for Clinical Laboratory Standards) and in previous reports.[6,7] The following procedure was used: lyophilised BCG Tokyo products were suspended in 1 ml of distilled water and were cultured on Middlebrook 7H10 agar (DIFCO, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC: BBL Prepared Culture Media, Becton Dickinson) at 37°C until sufficient growth was observed. After harvesting colonies from culture media, each lot strain of BCG Tokyo was dispersed by vortex mixing with glass beads (disperser tube: Nichibi, BCG Laboratory, Tokyo, Japan) and two drops of 10% Tween 80 (LC-MS, Santa Fe, CA, USA). After vortex mixing for 30 s, 1 ml of distilled water was added to each sample and they were vortexed again for 10 s. The supernatant of each bacterial suspension was transferred to 10 ml of Middlebrook 7H9 broth supplemented with albumin, dextrose and catalase (BBL Prepared Culture Media, Becton Dickinson), and the suspension density was adjusted to an optical density (OD) of 0.05 at 530 nm. These culture tubes were incubated at 37°C with daily mixing and OD checking. When the OD reached 0.2, they were used as the original bacterial suspension.

To prepare 10^{-2} dilutions, a 100 µl aliquot was transferred into 10 ml of distilled water. In a similar way, 100 µl of the 10^{-2} dilution was added to 10 ml of distilled water for 10^{-4} dilutions. One hundred microlitres of the 10^{-2} dilution were inoculated onto Middlebrook 7H11 agar plates with anti-tuberculosis drugs at the designated concentrations. Final INH concentrations were 0.03, 0.06, 0.125, 0.5, 1.0 and 2.0 µg/ml. RMP (0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 µg/ml), SM (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) and EMB concentrations (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) were adjusted accordingly. The 10^{-2} and 10^{-4} suspensions were inoculated onto Middlebrook 7H11 medium containing no drugs for growth control and 1% proportion measurements. These plates were incubated at 37°C. When the 10^{-2} dilution control showed sufficient growth (>100 visible colonies), the MICs were measured as the lowest concentration of drug that inhibited more than 99% of the bacterial population compared with the number of colonies on drug-containing media and the 10^{-4} growth control. Each test was performed in triplicate.

RESULTS

The MICs of one seed and five product lots were measured in triplicate. The MICs of the anti-tuberculosis drugs varied slightly with the lots tested, but were identical among the triplicate tests. The MICs for all tested drugs are shown in the Table. The MICs of INH were 0.06 µg/ml and the seed lot MIC was 0.125 µg/ml. The MIC in test 3 of lot A was not determined due to contamination. For RMP, the MICs for lots A, B and C were 0.25 µg/ml; those for lots D and E were 0.5 µg/ml. It was considered that the MICs of RMP were between 0.25 and 0.5 µg/ml. For SM, the MICs were determined to be 0.25 µg/ml in all tests. For EMB, the MICs were 4 µg/ml for lots A, B and C, while the MICs for lots D and E were 2 µg/ml. The MIC of EMB was 2–4 µg/ml.

DISCUSSION

The BCG vaccine was developed by Calmette and Guérin in 1921. All BCG vaccines consist of live attenuated *Mycobacterium bovis* bacteria. BCG vaccination is commonly performed on neonates and infants once or twice in middle to high tuberculosis prevalence countries, and more than 100 million children have received BCG in recent years.[8] Its safety is therefore a priority issue.

BCG vaccination may sometimes cause complications as a pathogen. Local adverse effects of BCG vaccination have at times been observed and usually improve spontaneously, although severe complications in immunocompromised patients have been reported. McKenzie et al. reported systemic haematological dis-

Table MIC values of four first-line drugs for the BCG Tokyo strain

	MIC (µg/ml)											
	INH			RMP			SM			EMB		
Samples	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Lot A	0.06	0.06	cont	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot B	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot C	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot D	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Lot E	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Seed lot	0.125	0.125	0.125	ND	ND	ND	ND	ND	ND	ND	ND	ND

MIC = minimum inhibitory concentration; BCG = bacille Calmette-Guérin; INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol; cont = contaminated; ND = not done.

semination of BCG in a child with X-linked severe combined immunodeficiency.[9] Puthanakit et al. reported four cases of BCG infection in HIV-positive children receiving BCG vaccinations at birth; the strain was not indicated.[10]

BCG strains have also been utilised for immunotherapy in addition to TB prevention. BCG is injected into the urinary bladder for intravesical instillation therapy in the early stages of bladder carcinoma.[11,12] The BCG Tokyo strain is popular for such adjuvant therapy in Japan,[13] whereas the Connaught strain is popular in other parts of the world. In a recent study, Mugiya et al. described good, complete response rates of 84% with BCG Tokyo (40 mg administered every 6 weeks) against bladder carcinoma in situ.[14] However, adverse reactions can also occur after instillation therapy. Eichel et al. reported INH-resistant BCG cystitis successfully treated with RMP and EMB.[15]

There is at present no recommended treatment regimen for BCG infection. Anti-tuberculosis drugs are the most potent agents for treating BCG infection. Drug susceptibility testing (DST) of BCG strains has been reported using different methods. Durek et al. evaluated the Connaught BCG strain using a BACTEC 460TB system (Becton Dickinson).[16,17] DST was performed for 31 drugs, including INH, RMP, SM, EMB and rifabutin. The BCG Connaught strain was susceptible to all of the anti-tuberculosis drugs except pyrazinamide (PZA) (BCG has natural/intrinsic resistance to PZA) and some other drugs used for general bacterial infections. The BACTEC 460 TB system employs critical drug concentrations of 0.1, 1.0, 2.0 and 2.5 for INH, RMP, SM and EMB, respectively. Rousseau and Dupuis reported the DST for a seed lot of the BCG Montreal strain by using solid Dubos medium.[18] They showed that this strain was sensitive to INH (0.2 μg/ml), RMP (1.0 μg/ml), SM (2.0 μg/ml) and EMB (5.0 μg/ml). These reports are not, however, comparable because of the differences in testing methods. There is no standard method for the DST of BCG; however, they may be equivalent to each other in the concept of detecting 1% resistance in the strain population. The proportion method with Middlebrook 7H11/OADC media, which is commonly used for the DST of *M. tuberculosis*, was used for this study.

The MICs indicated in the present study were lower than the critical concentrations employed in the previous studies, except for EMB with MIC close to the critical concentration of BACTEC. In the previous studies, the MICs of EMB to *M. tuberculosis* vary between 0.5 μg/ml and 2.0 μg/ml,[19,20] in 7H12 BACTEC broth MIC varies between 0.95 and 3.8 μg/ml and on 7H10 agar between 1.9 and 7.5 μg/ml.[21] Heifets proposed possible guidelines for the interpretation of MIC to *M. tuberculosis* determined in Middlebrook 7H12 broth (radiometric), and MIC 4.0 μg/ml of EMB as moderately susceptible.[22] It is possible that the MIC of BCG Tokyo strain for EMB was higher than wild type *M. tuberculosis*. However, these reports show the tendency of lower MIC in liquid media than solid media. The plasma concentration (C_{max}) of EMB reaches 2.0–5.0 μg/ml[23] and EMB generally works in a time-dependent manner. For this reason it is suggested that EMB could be effective. Although BCG and *M. tuberculosis* are different species, these MICs and pharmacokinetic data would support the potentials of EMB for the treatment of BCG infection. It was therefore considered that, like the BCG Montreal and Connaught strains, the BCG Tokyo strain is susceptible to the four major anti-tuberculosis drugs.

Hesseling et al. reported that BCG in an HIV co-infected infant who received a BCG Danish 1331 strain vaccination developed INH and RMP resistance following treatment with INH and RMP.[24] The MICs of the original strain were 0.15 and <0.4 μg/ml for INH and RMP, respectively. However, they had risen above 0.3 and 32 μg/ml after treatment. These results suggest that the strain was already clinically resistant to INH (MIC 0.15 μg/ml for INH), and monotherapy with RMP against BCG resulted in RMP resistance. Su et al. reported two general disseminated cases of the BCG Tokyo vaccine strains.[5] One of them was treated using anti-tuberculosis drugs (INH, RMP, SM and EMB) based on the susceptible DST results, and the patient recovered. Another case died following one month's treatment with INH, RMP and EMB. However, no DST data were shown in the mortality case and the infant seemed to have died from severe combined immunodeficiency. The MICs of the BCG Tokyo strain indicated in this study were considered less than or equivalent to those of the previous cases, so it was estimated that BCG Tokyo could be treated successfully even in severe adverse events such as systemic dissemination.

The reason why BCG strains have different phenotypic characteristics with respect to drug susceptibility is not clear. BCG has lost several regions of difference (RD) compared to *M. bovis* as the ancestral strain. In particular, the RD1 deletion made a significant contribution to the attenuation of BCG.[24–26] RD1 encodes a 6 kDa early secreted antigenic target protein (ESAT-6)[27] and a 10 kDa culture filtrate protein (CFP-10)[28] associated with virulence in *M. tuberculosis* complex. The BCG vaccine therefore has attenuated virulence compared to wild *M. bovis* strains. The loss of virulence apparently occurred through repeated passages.

The BCG strains were originally donated by the Pasteur Institute (Paris, France), and have been subcultured by several tuberculosis institutes around the world (Russia, Brazil, Sweden, Denmark, Japan, etc.) since 1924. The donated BCG strains differ from the original BCG strains due to differences in passage cultivation, culture medium and storage conditions. In 1972, Hesselberg found that a Swedish/Norwegian BCG strain became resistant to INH during the period 1953–1964, which was the reason why the serial sub-

culture system was discontinued and a seed lot system was adopted.[29] However, in 2003, low-grade INH-resistant (MIC >0.5 μg/ml) Danish-1331 strains were reported again to the World Health Organization (WHO). The WHO therefore recognises the necessity of a new quality assurance method for BCG vaccines.[30,31]

The BCG Tokyo strain was obtained from Calmette in the Pasteur Institute in 1924. Passage cultivation of BCG Tokyo strain has been performed strictly according to Calmette's original instructions, while some of the other BCG strain passages were tailored to each institute's needs. The BCG Tokyo 172 strain, which has undergone 172 passages since the Second World War II, has been used as the seed lot for lyophilised BCG Tokyo vaccines. In this study, the BCG Tokyo strain proved to be susceptible to the major anti-tuberculosis drugs; however, the results of this study do not apply to all BCG substrains. It will be necessary to ensure the safety of BCG vaccine by checking susceptibility to other antimicrobial agents.

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RÉSUMÉ

CONTEXTE : Le bacille de Calmette et Guérin (BCG) à base de *Mycobacterium bovis* est un vaccin unique contre la tuberculose (TB) en raison de ses effets protecteurs valables et de sa faible virulence. Toutefois, il peut causer occasionnellement une infection systémique chez les sujets en état d'immunodépression. L'isoniazide (INH), la rifampicine (RMP), la streptomycine (SM) et l'éthambutol (EMB) sont des médicaments antituberculeux reconnus comme efficaces et peuvent être utilisés dans le traitement des infections par le BCG. Il n'y a malheureusement que peu d'études concernant la sensibilité des souches de vaccin BCG à l'égard de ces médicaments.
OBJECTIF : Mesurer les concentrations minimales inhibitrices (CMI) du vaccin BCG Tokyo pour les médicaments antituberculeux et évaluer la sécurité du vaccin en ce qui concerne la sensibilité aux médicaments.
SCHÉMA : Nous avons mesuré les CMI sur plaques d'agar Middlebrook 7H11 pour la souche-mère et pour cinq lots de vaccin de la souche BCG Tokyo à la fois pour l'INH, la RMP, la SM et l'EMB.
RÉSULTATS : Les résultats des CMI pour l'INH ont été respectivement de 0,06 et de 0,125 μg/ml pour la souche-mère et pour les lots de vaccin. Les résultats des CMI pour la RMP, la SM et l'EMB ont été respectivement de 0,25–0,5, 0,25 et 2–4 μg/ml.
CONCLUSION : Nos résultats indiquent que la souche BCG Tokyo est sensible à l'égard des médicaments antituberculeux majeurs qui sont efficaces même en cas d'effets indésirables graves, y compris des infections systémiques.

RESUMEN

MARCO DE REFERENCIA : *Mycobacterium bovis*, el bacilo de Calmette y Guérin (BCG), es la única vacuna contra la tuberculosis (TB), debido a su valioso efecto de protección y a su baja virulencia. Sin embargo, esta vacuna puede causar en ocasiones infecciones generalizadas en individuos inmunodeprimidos. Isoniazida (INH), rifampicina (RMP), estreptomicina (SM) y etambutol (EMB) son medicamentos antituberculosos eficaces y se emplean en el tratamiento de las infecciones por BCG. Desafortunadamente, existen pocos estudios sobre la sensibilidad de la cepa de la vacuna antituberculosa a estos medicamentos.
OBJETIVO : Medir las concentraciones mínimas inhibitorias (CMI) de los medicamentos antituberculosos contra el BCG de Tokio contenido en las vacunas y evaluar su seguridad toxicológica en la concentración de sensibilidad al medicamento.
MÉTODOS : Se midieron las concentraciones inhibitorias mínimas de INH, RMP, SM y EMB para un lote de siembra y cinco lotes de vacuna de la cepa BCG de Tokio usando cultivos en placas de agar con Middlebrook 7H11.
RESULTADOS : La CMI para INH fue 0,06 con los lotes de siembra y 0,125 μg/ml con los lotes de vacuna. La CMI para los lotes de vacuna con RMP fue de 0,25 a 0,5 ; con SM fue 0,25 ; y con EMB fue de 2 a 4 μg/ml.
CONCLUSIÓN : Estos resultados indican que la cepa BCG de Tokio es sensible a los principales medicamentos antituberculosos y que es posible tratar los casos de reacciones adversas graves, incluida la infección generalizada.