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Expression of the 38 kDa Protein of *Mycobacterium tuberculosis* in *M. bovis* BCG and Use in the Serodiagnosis of Tuberculosis

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The 38 kDa protein of *Mycobacterium tuberculosis*, which was known previously as antigen 5, has been extensively used in the serodiagnosis of tuberculosis. In an attempt to develop and evaluate a serodiagnostic test using the antigen, we expressed the 38 kDa protein in BCG and its seroreactivity was compared to that expressed in *Escherichia coli*. The coding region of the 38 kDa protein was amplified by PCR, and the gene was cloned into a *Mycobacterium-E. coli* shuttle expression vector pYMC-*his* and pQE30 expression vector and expressed in BCG and *E. coli*, respectively. Both recombinant 38 kDa proteins showed strong seroreactivity against pooled serum from tuberculosis patients. There was no significant difference in seroreactivity between the two recombinant antigens in sera from the far advanced tuberculosis patients. However, of 25 tuberculosis patients graded as "minimal" by chest X-ray, 5 (20.0%) were seropositive by r38 kDa expressed in *E. coli*, while 8 (32.0%) by that expressed in BCG. Likewise, higher seroreactivity by r38 kDa expressed in BCG was found in sera from the moderately advanced tuberculosis. This study thus indicates that the recombinant 38 kDa expressed in BCG is more effective than that expressed in *E. coli* in detecting antibodies to the native 38 kDa protein of *M. tuberculosis* in sera from minimally affected tuberculosis patients.

Key Words: Tuberculosis, Serodiagnosis

INTRODUCTION

Tuberculosis is still one of the major public health problems worldwide. About one-third of world populations are infected with *Mycobacterium tuberculosis*, and more than 3 millions die due to tuberculosis each year (3). Major control strategies against tuberculosis consists of early detection, effective chemotherapy, and BCG vaccination. With continuing controversy over effectiveness of BCG vaccine against tuberculosis (8), early case detection becomes more important than ever.

Current approach of new case detection of tuberculosis relies mainly on passive case finding in most of the countries with high prevalence of tuberculosis. Although miniature chest X-ray screening has been used in some countries including Korea, its effectiveness has not been appreciated. Therefore, new screening test with greater sensitivity than the miniature chest X-ray would be great value for tuberculosis control programs worldwide.

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Among new diagnostic tests under development, serological tests have brought most attention because of its simplicity and low cost as well as high sensitivity. One of the most widely used antigens is the 38 kDa protein of *M. tuberculosis*, which has been known as antigen 5 (1) and glycosylated (2). The gene encoding the 38 kDa protein had been cloned and expressed in *E. coli* for use in serological tests (7, 10). Since recombinant proteins expressed in *E. coli* could not be glycosylated, we attempted to express the 38 kDa protein in BCG using the *Mycobacterium-E. coli* shuttle vector pYMC-*his* (5) in this study. In addition, the seroreactivity between 38 kDa proteins expressed in BCG and in *E. coli* was compared using a set of serum samples from tuberculosis and controls.

MATERIALS AND METHODS

Organisms. *Mycobacterium tuberculosis* H37Rv was obtained from Patrick J. Brennan (Mycobacterial Research Laboratory, Colorado State University, Fort Collins, Colo.) and maintained in the Ogawa media. *Mycobacterium bovis* BCG, Pasteur strain, was obtained from the Korea Institute of Tuberculosis, Seoul, Korea and also maintained in the Ogawa media. *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, Calif.) and *E. coli* strain M15[pREP4] (Qiagen, Chatsworth, Calif.) were purchased commercially and maintained in LB agar (Difco, Detroit, Mich.).

Expression of 38 kDa protein of *M. tuberculosis*. The recombinant techniques described by Sambrook *et al.* (6) were employed for cloning and expression with minor modification. First, the complete DNA sequence of the gene encoding the 38 kDa protein of *M. tuberculosis* was amplified from *M. tuberculosis* H37Rv chromosomal DNA by polymerase chain reaction (PCR) using *Taq* DNA polymerase in a thermocycler. The primers used in PCR amplification were derived from DNA sequences of the gene encoding the 38 kDa protein in the gene bank (accession No.: M30046), and the restriction enzyme sites were added to facilitate cloning. The PCR products were then ligated to pT7Blue® vector (Novagen, Madison, Wis.) resulting in pT7Blue:38 kDa and used for transformation of *E. coli* XL1. The pT7Blue: 38 kDa was prepared from the transformed *E. coli* and digested with restriction enzymes. The 38 kDa gene insert was cloned into the *Mycobacterium-E. coli* shuttle expression vector, pYMC-*his* (5), resulting in pYMC-*his*:38 kDa. The competent cells of *M. bovis* BCG were transformed with pYMC-*his*:38 kDa, and transformants were selected on Middlebrook 7H10 agar containing 25 μg/ml of kanamycin. After confirming the production of the 38 kDa protein with heat induction, the expressed proteins were purified using Ni^{+}-NTA resin. Likewise, the 38 kDa protein was expressed in *E. coli* using pQE30 vector system (Qiagen) and recombinant protein was purified using the Ni^{+}-NTA resin.

Detection of antibodies to recombinant 38 kDa proteins. An enzyme-linked immunosorbent assay (ELISA) described by Voller *et al.* (9) was employed with minor modification. Briefly, the r38 kDa protein was diluted in carbonate buffer, pH 9.6 to reach 1.0 μg/ml, and a volume of 100 μl was added to each well of 96-well flat bottom EIA plate (Costar Co., Cambridge, Mass.) and incubated at 4℃ overnight. After washing the well four times with phosphate-buffered solution containing 0.05% Tween 20 (PBST), pH 7.4, 200 μl of PBST containing 10% normal goat serum (NGS) (Gibco-BRL, Gaithersburg, Md.) was added to the wells, and the plates were placed in a moist chamber and incubated at 37℃ for one hour. After emptying the wells, 100 μl of serum diluted 1: 300 in PBST-NGS was added to each well and incubated at 37℃ for 90 min. The wells were then washed four times with PBST, and 100 μl of

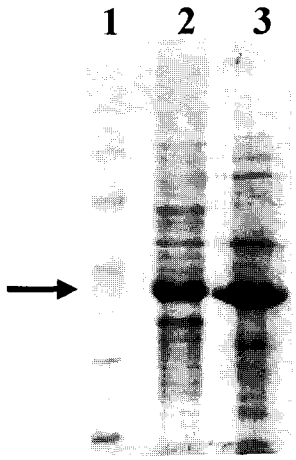

Figure 1. SDS-PAGE analysis of the recombinant 38 kDa proteins. The arrow indicates the recombinant 38 kDa proteins. Lane 1: size marker (97, 66, 45, 31, and 21 kDa), Lane 2: BCG-r38 kDa, and Lane 3: EC-r38 kDa.

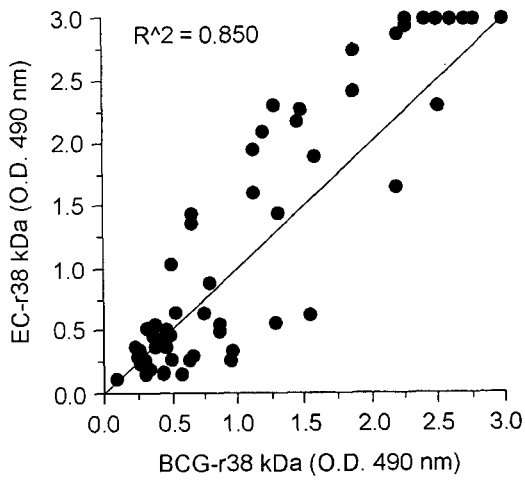

Figure 2. Comparison between IgG seroreactivity to BCG-r38 kDa and EC-r38 kDa in sera from tuberculosis patients. Each dot indicates an individual tuberculosis patient.

horseradish peroxidase conjugated anti-human IgG (Calbiochem, San Diego, Calif.) diluted 1: 20000 in PBST-BGS was added to the wells and incubated at 37℃ for one hour. After final washing four times with PBST, 100 μl of the substrate solution containing *O*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) and hydrogen peroxide was added to the wells and incubated at room temperature in a dark chamber for 15 min. After stopping the reaction with 100 μl of 2.5 N H_2SO_4, the optical density was read by an automatic ELISA reader (Molecular Devices Co., Sunnyvale, Calif.). Each serum was assayed in duplicate, and the mean absorbance of carbonate buffer control wells was subtracted from the mean absorbance of the r38 kDa protein-coated wells before analysis.

RESULTS AND DISCUSSION

Recombinant 38 kDa proteins expressed in *M. bovis* BCG (BCG-r38 kDa) and *E. coli* (EC-r38 kDa) were analyzed by SDS-PAGE, and the results were shown in Figure 1. The BCG-r38 kDa protein was slightly greater in size when determined by electrophoretic mobility in polyacrylamide gel, but it was not known yet if the BCG-38 kDa was glycosylated. Both recombinant proteins were reactive with sera from tuberculosis patients in western blotting analysis (data not shown). The results thus indicated that recombinant 38 kDa proteins expressed in BCG and *E. coli* were similar in molecular size and had antigenicity.

In order to compare seroreactivity between

Table 1. Comparison between r38 kDa proteins expresssed in *E. coli* and BCG for the detection of antibodies from tuberculosis patients

Chest X-ray Gradings	No. assayed	Seroreactive[a] to r38 kDa from *E. coli* No. (%)	Seroreactive[a] to r38 kDa from BCG No. (%)
Minimal	25	5 (20.0)	8 (32.0)
Moderately advanced	19	12 (63.2)	16 (84.2)
Far advance	8	7 (87.5)	7 (87.5)
Controls (Korea)	100	5 (5.0)	7 (7.0)
Controls (U.S.)	30	0	ND

[a]Criteria : O.D. 490 nm> 0.75 for *E. coli* r38 kDa; 0.65 for BCG r38 kDa. ND: not done.

BCG-r38 kDa and EC-r38 kDa, a set of serum samples from tuberculosis were examined for seroreactivity to both proteins. As shown in Figure 2, most of the serum samples showed similar seroreactivity to both recombinant 38 kDa proteins (R^2=0.850). However, serum samples with lower seroreactivity had a tendancy to give greater reactivity to BCG-r38 kDa. It was not known whether or not the higher seroreactivity to BCG-r38 kDa protein were due to glycosylation.

When the ELISA absorbance of 0.75 and 0.65 were used as the cut-off criteria for seropositivity to EC-r38 kDa and BCG-r38 kDa, respectively, there was no significant difference in sera from the far advanced tuberculosis patients (Table 1). However, there was clear difference in seroreactivity between the two recombinant proteins in sera from patients with minimal and moderately advanced patients. Of 25 tuberculosis patients with minimal involvement in chest X-ray, 8 (32%) were seropositive to BCG-r38 kDa protein compared to 5 (20.0%) to EC-r38 kDa. Likewise, 16 (84.2%) of 19 moderately advanced patients were seropositive to BCG-r38 kDa compared to 12 (63.2%) to EC-r38 kDa. There was no significant difference in overall specificity of the two recombinant proteins.

Therefore, these results indicated that BCG-r 38 kDa might be more effective than EC-r38 kDa in detecting antibodies in sera from patients with low level of anti-38 kDa antibodies, probably during the early stage of tuberculosis. Although this phenomenon may be due to glycosylation of the BCG-r38 kDa, we could not rule out the possibility of contamination of other *M. bovis* BCG antigens in the purified BCG-r38 kDa. Further study needs to address evidence of glycosylation in BCG-r38 kDa protein and the role of its carbohydrate moiety in seroreactivity if any.

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