Identification and characterization of serologically active mycobacterial antigens are prerequisites for the development of diagnostic reagents. We examined the humoral immune responses of active tuberculosis (TB) patients against Triton-soluble proteins extracted from *Mycobacterium tuberculosis* by immunoblotting. A 29-kDa protein reacted with immunoglobulin M (IgM) in the pooled sera of the patients, and its N-terminal amino acid sequence matched that of the heparin-binding hemagglutinin (HBHA). Recombinant full-length HBHA was expressed in *Escherichia coli* (rEC-HBHA) and *M. smegmatis* (rMS-HBHA). In immunoblot analysis, the IgM antibodies of the TB patients reacted strongly with rMS-HBHA but not with rEC-HBHA, whereas the IgG antibodies of these patients reacted weakly with both recombinant HBHA proteins. In enzyme-linked immunosorbent assay analysis using rMS-HBHA and 85B as antigens, the mean levels and sensitivities of the anti-HBHA IgM antibodies of the TB patients were significantly higher than those of the anti-antigen 85B IgM antibodies, while the IgG antibodies showed the opposite results. Of interest in this respect, the pooled sera from the TB patients that contained anti-HBHA IgM antibodies neutralized the entry of *M. tuberculosis* into epithelial cells. These findings suggest that IgM antibody to HBHA may play a role in protection against extrapulmonary dissemination.

**MATERIALS AND METHODS**

**Human sera.** Sera were obtained from TB patients and healthy controls. Informed consent was obtained before blood was drawn. The patients with pulmonary TB were divided into early (no. = 33) and chronic (no. = 21) groups. The chronic patients had been admitted to the National Mokpo Tuberculosis Hospital (Mokpo, Chonnam, Korea) and had received therapy for over 12 months. The early group consisted of outpatients at the Chungnam National University Hospital (Daejeon, Korea) who had received standard chemotherapy for less than 1 month. A diagnosis of TB was based upon clinical evaluation, sputum smear and culture, and/or chest X-ray results. The healthy control sera were obtained from 533 students at the Chungnam National University (Daejeon, Korea) who had no previous history of clinical TB. None of the subjects had any previous history of diabetes mellitus or steroid therapy, and all were human immunodeficiency virus negative.

**Culturing of *M. tuberculosis*, *M. tuberculosis* H37Rv (ATCC 27294), *M. fortuitum* (ATCC 6841), *M. kansasii* (ATCC 12478), *M. avium* (ATCC 19075), and *M. bovis* BCG (France) cultures were grown at 37°C as a surface pellicle on Sauton’s
medium. The bacilli and culture filtrate were prepared as previously described (9). The protein concentrations were determined using a protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

For the invasion assay, *M. tuberculosis* was grown at 37°C in roller bottles that contained Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 0.05% Tween 80 and 10% oleic acid-albumin-dextrose-catalase until the optical density at 600 nm (OD600) reached 0.5. The cells were collected by centrifugation, washed, resuspended in basal RPMI 1640, and centrifuged at 150 × g for 5 min to remove any clumps. Aliquots of the upper bacterial suspension were stored at −80°C until use. Quantification of the bacteria was performed by assessing the CFU after plating serial 10-fold dilutions of the supernatant onto Middlebrook 7H10 agar (Difco).

**Preparation of Triton-soluble protein extracts.** The aqueous phases of the Triton X-114 extracts from the mycobacteria were prepared as previously described (9).

**Fractionation of Triton-soluble proteins.** The Triton-soluble proteins were fractionated in a preparative isoelectric focusing (IEF) apparatus (Rotofor Cell; Bio-Rad, Hercules, CA). The protein sample (60 mg), ampholyte pH 3/10 (2% [wt/vol]), and ampholyte pH 4/6 (0.2%) were added in 3 M deionized urea. The 50-ml mixture was loaded into the IEF apparatus, and constant power (12 W) was applied while the system was cooled to 4°C. After focusing was performed, 20 fractions were collected. Individual fractions were dialyzed using 1 M NaCl to remove the ampholytes and finally dialyzed against phosphate-buffered saline (PBS).

**SDS-PAGE, immunoblotting, and identification of proteins.** The protein samples were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
phoresis (SDS-PAGE) with 12% polyacrylamide resolving gels and the discontinuous buffer system of Laemmli (10). Immunoblotting was performed as previously described (9). In brief, the blots were incubated with polyclonal antibodies (1:1,000) or pooled patient sera (1:200) diluted in blocking solution and then incubated with the appropriate peroxidase-conjugated secondary antibody (anti-human IgG, anti-human IgM, and anti-mouse IgG; Sigma-Aldrich, St. Louis, MO).

For N-terminal sequencing, each sample was subjected to electrophoresis and electrotransfered to polyvinylidene difluoride (PVDF) membranes. N-terminal sequencing was performed by the Edman degradation method at the Korea Basic Science Institute (Daejeon, Korea).

Partial purification of HBHA. The protein that reacted strongly with IgM in the patients’ sera was identified as a HBHA. Therefore, the protein was purified partially by heparin-Sepharose chromatography as described by Menozzi et al. (16).

Expression and purification of recombinant proteins. To produce recombinant HBHA (rHBHA) in Escherichia coli (E. coli-HBHA), the corresponding genes were PCR amplified using M. tuberculosis H37Rv DNA as the template and the following oligonucleotide primers: F primer (5′-AGGAGGATCCATGCTGGAAAACCTGACAC-3′) and R primer (5′-ATTCTGAAATTCGACTACTTCTGGGTGAC-3′). The forward and reverse primers contained the BamHI and EcoRI restriction sites (underlined), respectively. The PCR products were digested with BamHI and EcoRI and cloned into the pET21a vector (Novagen, Madison, WI), and the resulting clones were sequenced. E. coli strain BL21(DE3), which expresses the T7-tagged protein, was grown in Luria-Bertani broth supplemented with 100 μg/ml ampicillin. After induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), the cells were harvested by centrifugation, suspended in phosphate-buffered saline that contained 0.1 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication. The rE.C-HBHA was purified by heparin-Sepharose chromatography and then was further purified using a T7-T antigen (T7-Tag) affinity purification kit (Novagen) in accordance with the manufacturer’s instructions (16).

Recombinant HBHA was also expressed in M. smegmatis (mMS-HBHA). The pMV3-38 plasmid (pMV206-based construct) that contains the full-length HBHA open reading frame was kindly provided by G. Delogu (University of Sassari, Sassari, Italy) (4). The preparation of M. smegmatis competent cells and electroporation procedures were performed as previously described (2). The M. smegmatis (pMV3-38) recombinant strain that expresses the histidine-tagged HBHA was grown for 4 days at 37°C in Sauton’s medium containing 50 μg/ml hygromycin. The cells were harvested by centrifugation, the pellet was resuspended in 10 mM potassium phosphate buffer (PB; pH 7.0) that contained 4 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml lysozyme, and the cells were lysed by sonication on ice. After centrifugation, the supernatant was applied to a 300-μl phosphocellulose column (Whatman P11) equilibrated with 10 mM PB. The column was washed with 150 mM PB, and the bound materials were eluted with 750 mM PB. The eluates were concentrated, dialyzed using 20 mM Tris-HCl (pH 8.0) that contained 500 mM NaCl and 20 mM imidazole, and further purified by nickel-nitri triacetic acid (Ni-NTA) agarose chromatography in accordance with the manufacturer’s instructions (Invitrogen, Carlsbad, CA).

All of the purification steps were analyzed by SDS-PAGE and Coomassie blue staining and by immunoblotting using pooled patient sera.

Antibody production. To obtain antiserum to rEC-HBHA, BALB/c mice were immunized intraperitoneally with 25 μg of purified rEC-HBHA emulsified in incomplete Freund’s adjuvant. The mice were injected with the antigen three times at intervals of 2 weeks. The mice were sacrificed 1 week after the final immunization, and the serum was collected and stored at −70°C.

ELISA. Antigen 85B was purified by traditional biochemical chromatography, and the antibody titers were determined by an enzyme-linked immunosorbent assay (ELISA), as previously described (11). In brief, 96-well microplates (Falcon Pro-bind assay plate) were coated with antigen 85B (200 ng/well) and rMS-HBHA (200 ng/well) in 0.05 M carbonate buffer (pH 9.6) solution. The solution samples were diluted 1:80, and 0.1 ml was added to each well. After washing, 0.1 ml of peroxidase-conjugated goat anti-human IgG and anti-human IgM (Sigma-Aldrich) diluted 1:3,000 was added to each well.

A549 cell invasion assay. To examine the potentially protective role of human anti-HBHA IgM, the neutralizing activities of these antibodies, in terms of inhibiting bacterial attachment to and invasion of epithelial cells, were analyzed. Aliquots of M. tuberculosis were thawed, dispersed using a bath sonicator, and centrifuged at 150 × g for 2 min, and the supernatant was used for infection. The bacteria were incubated with pooled human sera at the indicated concentrations for 2 h at room temperature. The human sera used included pooled TB patient sera with high or low levels of anti-HBHA IgM (≥0.2 or <0.2 at OD405 from the ELISA data, respectively) as well as healthy control sera. The human type II alveolar pneumocyte cell line A549 (ATCC CCL185) was cultured and maintained in antibiotic-free RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) with 10% fetal bovine serum (GIBCO-BRL). For the assay, A549 cells were seeded onto 24-well plates at a density of 4 × 10⁵ cells/well 3 to 4 days before use. The assay was performed in triplicate. The cells were infected at a multiplicity of infection of 1 to 3 with M. tuberculosis preincubated with human sera, as described above, for 3 h at 37°C in 5% CO₂. Amikacin (200 μg/ml) was then added for 1 h to kill extracellular bacteria. The monolayers were washed three times with basal RPMI 1640 medium, and viable intracellular bacteria were released by incubation with 0.5 ml of 0.1% Triton X-100 in sterile water for 10 min. The samples were mixed vigorously with 0.5 ml of 7H9 broth. Serial 10-fold dilutions of the lysates were prepared in 7H9 broth and plated on 7H10 agar for determination of CFU numbers.

Statistical analysis. Duncan’s multiple range test was used to compare three samples; a P value of <0.05 was considered to be statistically significant. Student’s t test was used to compare the two groups.

FIG. 3. Analysis of Triton X-114 extracts by preparative IEF and determination of the N-terminal sequence of the 29-kDa protein. The aqueous phases of the Triton extracts were separated into 20 fractions by preparative IEF with the Rotofor cell. Each fraction was analyzed by SDS-PAGE (A) and immunoblotting (B). The gels were stained with Coomassie blue. The PVDF membrane was incubated with patient sera and visualized with anti-human IgM. Lane 1, pooled fractions from fractions 1 to 3; lanes 2, fraction 5; lanes 3, fraction 6; lanes 4, fraction 7; lanes 5, pooled fractions 11 and 12; lanes 6, pooled fractions 17 and 18; lanes 7, pooled fractions 19 and 20; lanes 8, Triton X-114 extract, aqueous phase.
RESULTS

Identification of the \textit{M. tuberculosis} protein that reacts with IgM from TB patients. Previously, we reported the preparation of \textit{M. tuberculosis} antigens by use of Triton X-100 (9). Since many of these antigens are likely to be associated with the mycobacterial envelope, this fraction may contain good targets for the induction of humoral immune responses. Therefore, the serological reactivities to the aqueous fraction of the Triton X-114-soluble \textit{M. tuberculosis} proteins were screened by immunoblotting using pooled TB patient sera. As expected, many bands reacted either strongly or weakly with the IgG from TB patients (Fig. 1B and Fig. 2). Interestingly, a single protein band of approximately 29 kDa reacted strongly with IgM (Fig. 1C); the pattern of immunoreactivity was similar to that seen for a specific monoclonal antibody (MAb).

To evaluate diagnostic utility, the presence in different mycobacterial species of the 29-kDa protein was examined at the protein level by immunoblotting with human sera. The aqueous phases of Triton-soluble extracts were prepared from several mycobacterial strains available in our laboratory. Many of these proteins reacted with the IgG of TB patients (Fig. 2). However, the 29-kDa protein that reacted with IgM in the sera of TB patients was present only in the aqueous fractions of \textit{M. tuberculosis} and \textit{M. bovis} BCG. While the TB patient IgG strongly recognized several proteins in 6-week-old culture filtrates of \textit{M. tuberculosis}, the 29-kDa protein reacting with IgM was not detected in the culture filtrates (Fig. 2, lanes 2).

Preparative IEF using Rotofor cells was performed to separate the aqueous fraction of the Triton-soluble proteins. The proteins were separated into 20 fractions based on isoelectric point (pI). The protein concentrations were very low in both side fractions of the pH range. Therefore, two or three contiguous side fractions were pooled and concentrated, and the ampholytes were removed by dialysis. Most of the proteins were found in fractions 4 to 9 (pH of 3 to 6) (Fig. 3). However, the 29-kDa protein was present in pooled fractions 19 and 20, which indicated a basic protein with pI of 10.

The 29-kDa band was excised from a PVDF membrane after SDS-PAGE, and the amino acid sequence of its N terminus was identified as AENSNIDDIK (Fig. 3). This sequence is identical to that of HBHA (14, 16).

Serological characterization of HBHA. HBHA is both a well-characterized adhesion molecule that is involved in the mycobacterium-epithelial cell interaction (20) and an immunoreactive protein (24). There are no reports that HBHA reacts specifically with TB patient IgM. To analyze the serological reactivities, purification of native HBHA from \textit{M. tuberculosis} sonicated extracts was performed by heparin-Sepharose chromatography, as described by Menozzi et al. (16). However, the protein could not be purified to homogeneity (data not shown).

The full-length \textit{M. tuberculosis} HBHA was expressed using \textit{E. coli} (Fig. 4A) and the pET21a expression vector and purified by heparin-Sepharose chromatography and T7-Tag affinity chromatography (Fig. 4A, lane 3). HBHA was also expressed using \textit{M. smegmatis} and the pmV206-based vector. Based on the basic pI of HBHA, rMS-HBHA was purified from the \textit{M. smegmatis} lysate by phosphocellulose chromatography, i.e.,

<table>
<thead>
<tr>
<th>Coomassie blue staining</th>
<th>Immunoblotting</th>
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<tr>
<td>kDa</td>
<td>M</td>
</tr>
<tr>
<td>92</td>
<td>113</td>
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<td>53</td>
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<td>36</td>
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FIG. 4. Immunoblot analyses of HBHA expression in \textit{E. coli} and \textit{M. smegmatis}. (A) Recombinant full-length HBHA protein was expressed in \textit{E. coli}. The rEC-HBHA protein was purified by heparin-Sepharose and T7-Tag affinity chromatography. The lysates of an uninduced culture (lane 1) and IPTG-induced culture (lane 2), as well as purified rEC-HBHA protein (lane 3), were analyzed. (B) His-tagged recombinant HBHA (rMS-HBHA) was expressed in \textit{M. smegmatis} and purified by Ni-NTA agarose chromatography (lane 4). (C) Purified Ag85B protein from the culture filtrate of \textit{M. tuberculosis} (lane 5). The antibody reactivities to purified rEC-HBHA (lanes 3), rMS-HBHA (lanes 4), and partial purified native HBHA (lane 6) were analyzed by immunoblotting with mouse anti-rEC HBHA antibody (D), TB patient IgM (E), and TB patient IgG (F).
TABLE 1. Comparison of IgG and IgM antibody responses to rMS-HBHA and Ag85B proteins in TB patients and healthy controls

<table>
<thead>
<tr>
<th>Patient group (no. of subjects)</th>
<th>ELISA result for rMS-HBHA</th>
<th>ELISA result for Ag85B</th>
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<tbody>
<tr>
<td></td>
<td>rMS-HBHA</td>
<td>Ag85B</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
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<tr>
<td></td>
<td>Mean OD ± SD</td>
<td>No. (%) of positive serum samples</td>
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<tr>
<td>Chronic TB (21)</td>
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<tr>
<td></td>
<td>0.23 ± 0.15‡</td>
<td>8 (38)</td>
</tr>
<tr>
<td>Early TB (33)</td>
<td>0.15 ± 0.07§</td>
<td>5 (15)</td>
</tr>
<tr>
<td>Healthy controls (33)</td>
<td>0.08 ± 0.05§</td>
<td>1 (3)</td>
</tr>
</tbody>
</table>

Sera were collected both from TB patients who had been treated for >1 year (chronic TB) and <1 month (early TB) and from healthy controls and were diluted 1:80.

Values are expressed as means of OD_{450} ± SD.

The cutoff value for IgG and IgM antibodies to each antigen was calculated as the mean OD obtained with sera from 33 healthy controls plus 3 SD.

Duncan’s test for multiple comparisons of means was used to determine whether there were significant differences between the results for the three groups. The different symbols (†, ‡, §) indicate significant differences (P < 0.05) between the means of antibody titers for the three groups.

HBHA (data not shown). These results indicate that an epitope recognized by human IgM is not present in the recombinant protein produced by E. coli.

**TB patient serum immunoreactivity with rMS-HBHA.** We characterized the serological reactivities to HBHA of 21 chronic TB and 33 early TB patients, as well as of 33 healthy controls, by measuring the levels of serum IgG and IgM antibodies to rMS-HBHA and antigen 85B in ELISAs (Table 1). Figure 4C shows the purification of antigen 85B, as previously described (11). The IgG and IgM antibody responses to two antigens in chronic TB patients were significantly higher than those in early TB patients and healthy controls (P < 0.05).

The levels of IgG antibody to both antigens in the early TB group were significantly higher than those in the control group, although no significant differences in the mean levels of IgM antibody to both antigens were noted between the early TB and control groups. The mean levels of IgM antibody to rMS-HBHA in the patient groups were significantly lower than the mean levels of IgM antibody to antigen 85B, while the titers of IgG antibody showed the opposite results (Table 1).

Using a cutoff value greater than the mean plus 3 standard deviations (SD) of the results obtained with the healthy controls, the sensitivities of the anti-antigen 85B IgG antibodies in both TB patient groups were higher than those of the anti-rHBHA IgG antibodies, whereas the sensitivities of the anti-rHBHA IgM antibodies were significantly higher than those of anti-antigen 85B IgM antibodies (Table 1). When the individual results were scrutinized, it was noted that some of the sera that were negative for IgG were positive for IgM. Combining the results for IgM and IgG against rMS-HBHA increased the sensitivity to 52% and 24% in the chronic TB and early TB groups, respectively.

**Effects of TB patient sera on the ability of M. tuberculosis to attach to A549 cells.** It has been reported that the binding of *M. tuberculosis* to epithelial cells is inhibited by anti-HBHA MAbs (16). Therefore, we investigated whether TB patient sera could inhibit the invasion of *M. tuberculosis* into A549 cells. The pooled TB patient sera at 5-, 10-, and 50-fold dilutions inhibited bacterial invasion significantly better than did the healthy control sera (Fig. 5). Invasion was inhibited by the patients’ sera in a dose-dependent manner. The sera of TB patients with higher levels of IgM against HBHA inhibited invasion significantly better than did the sera of TB patients with lower levels of IgM (P < 0.05). These results suggest that IgM antibody to HBHA in the serum may neutralize the attachment of *M. tuberculosis* to epithelial cells.

**DISCUSSION**

*M. tuberculosis* HBHA is a surface-associated protein that mediates bacterial attachment to epithelial cells (16) and has been shown to be required for the extrapulmonary dissemination of these bacteria (20). In this study, we found that this protein binds strongly to the IgM of TB patients and that
patient sera that contained anti-HBHA IgM antibodies neutralized the attachment and invasion of \textit{M. tuberculosis} to epithelial cells.

Menozzi et al. (16) reported the purification of HBHA from culture supernatants of \textit{M. tuberculosis} and \textit{M. bovis}; however, we were unable to detect HBHA in culture filtrates by immunoblotting with pooled sera from TB patients. HBHA, which does not contain a signal sequence for secretion, may be produced due to degradation of the cell or shearing from the membrane. In our experience, HBHA is a very unstable protein that is rapidly degraded and lost during multiple purification steps. The culture filtrates used in the present study were prepared from 6-week-old cultures, and an additional week was required for protein concentration. Thus, degradation during processing may be one of the reasons that we could not detect HBHA in the culture filtrates.

The HBHA protein that reacted strongly with the IgM of TB patients was also present in the \textit{M. bovis} BCG extract but not in that of \textit{M. avium} or \textit{M. kansasii}. This finding is in agreement with previous reports of HBHA production by \textit{M. bovis} and \textit{M. tuberculosis} (14, 16). \textit{M. avium} produces a protein with 89% similarity to \textit{M. tuberculosis} HBHA at the amino acid level (17, 22). It is possible that the epitopes recognized by the IgM from the TB patients were not present in the HBHA produced by \textit{M. avium}. Another possibility is that the level of HBHA-related protein in the \textit{M. avium} extract was too low to allow recognition by the IgM antibody.

It has been demonstrated that HBHA undergoes a process of methylation involving the lysine residues present at the C terminus that provides important biological properties (14, 18, 19, 24). In this study, a discrepancy of molecular weight between rEC-HBHA and rMS-HBHA was observed. It is most likely due to methylation of rMS-HBHA that is not performed when the protein is produced by \textit{E. coli} (14). Two MAbs have been identified: MAb E4, which reacts with the native HBHA and rEC-HBHA, and MAb D2, which reacts exclusively with the native HBHA (5). Although both MAbs recognize a KAP repeat region at the C terminus of HBHA, methylation of lysine residues is required for reactivity with MAb D2. In the present study, we found that human IgM antibody could effectively recognize rHBHA expressed by \textit{M. smegmatis} but could not recognize rHBHA expressed by \textit{E. coli}. Taking previous reports into consideration, our results suggest that the human anti-HBHA IgM antibody recognizes the methylated lysine repeat motifs of HBHA.

It was previously reported that patients with tuberculosis produced anti-HBHA IgG much more frequently than did the infected healthy subjects (13). Since IgM antibodies appear rapidly following infection, they are expected to be of diagnostic value in cases of recently acquired tuberculosis. However, with respect to IgM detection, sensitivity is low. Therefore, IgG-specific assays have been traditionally used. In the present study, the sensitivity of the IgM antibody to antigen 85B was very low compared to the IgG sensitivity of 95% in the chronic TB patient group. Kalantri et al. (8) have reported IgG and IgM sensitivities of 80% and 28.5%, respectively, for an ELISA that used A60. Another report indicated an IgG sensitivity of 62% and an IgM sensitivity of only 11% for an assay that used the 26-kDa antigen (21). In contrast, the sensitivities of the IgM antibodies against HBHA in the chronic TB and early TB patients were very high compared to the sensitivities of the IgM antibodies against antigen 85B. Recently, Zanetti et al. (26) have reported that IgG antibody titers against rMS-HBHA in TB patients are higher than those against antigen 85B and other antigens. However, in our study, the mean level of IgG antibody to antigen 85B was higher than that of IgG antibody to rMS-HBHA. This discrepancy is probably due to differences in immunogenetics between subject populations and in the antigens used. Recombinant antigen 85B was used in the previous study, whereas we used the native 85B antigen; the same rMS-HBHA was used in both studies.

Although numerous studies have focused on the cellular mechanisms responsible for protection against mycobacterial infection, little is known about the possible role of antibodies in TB. HBHA binds to epithelial cells, but not to professional phagocytes, via its C-terminal lysine-rich domain. An HBHA-deficient strain has been shown to be defective in extrapulmonary dissemination from the lungs (20). Moreover, the dissemination of wild-type \textit{M. tuberculosis} can be inhibited by preincubation with MAbs that recognize the HBHA C-terminal methyl-lysine motifs. The present study demonstrates that human anti-HBHA IgM antibodies recognize the methylated lysine motifs of HBHA and may block the binding via these motifs of \textit{M. tuberculosis} to epithelial cells, as the pooled sera from TB patients with higher IgM titers more effectively inhibited the invasion of bacteria into A549 cells than did the pooled sera from TB patients with lower IgM titers. However, we cannot exclude protective roles for IgG antibodies against HBHA or for antibodies against other mycobacterial components. Although HBHA is the predominant factor in \textit{M. tuberculosis} binding to respiratory epithelial cells, there are other proteins involved in this binding (23). In addition, the polarized epithelial cell model is more reliable for assays of invasion of the bacteria into the cells than the epithelial cell monolayer model applied to our study. However, it has already been reported that HBHA-coupled gold particles are transcytosed across an epithelial layer made of polarized pneumocytes (15). Taken together, our findings suggest that IgM antibodies to HBHA play a role in protection against extrapulmonary dissemination of \textit{M. tuberculosis}.

**ACKNOWLEDGMENTS**

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