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Patients with Pulmonary Tuberculosis Develop a Strong Humoral Response against Methylated Heparin-Binding Hemagglutinin

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Reactivities of human sera against selected recombinant *Mycobacterium tuberculosis* antigens were assessed by enzyme-linked immunosorbent assay. The results obtained indicate that patients with tuberculosis (TB) do not develop a strong humoral response against PE_PGRS and PPE proteins or against the Ag85B and heparin-binding hemagglutinin (HBHA) recombinant antigens. Conversely, purified methylated HBHA was strongly recognized by sera obtained from TB patients compared to controls.

Tuberculosis (TB) is a major infectious disease that kills almost 2 million people every year, mostly in developing countries, where it poses a major health, social, and economic burden (16). The development of improved, clinically sensitive, rapid, and economical diagnostic tests may provide a powerful tool to better control the epidemic. Recently, PCR-based methods and automatic culture systems have been made available, and these methods are in extensive regular use in laboratories in developed countries (1, 3). Moreover, these diagnostic systems are not suited for field use. The idea to develop a test for the diagnosis of TB through a serological assay has been pursued for many decades, but the results so far have been poor. Many of the antigens did not have an adequate sensitivity or specificity, and these assays could not properly distinguish *Mycobacterium bovis* BCG-vaccinated and purified protein derivative (PPD)-positive individuals from those with active TB.

Recently, it has been demonstrated that several mycobacterial proteins undergo a process of posttranslational modification in mycobacteria that provides important immunological properties (15). Among these proteins is heparin-binding hemagglutinin (HBHA), which undergoes a process of methylation involving the lysine residues present at the C terminus (13). Since the recombinant proteins obtained in *Escherichia coli* cannot be properly processed, the use of these antigens in serological assays is precluded by the cumbersome procedures required to purify the native antigens. Recently, a rapid and effective system for the purification of methylated HBHA has been developed, and the use of these proteins in such assays is now feasible (5).

In this study, the humoral responses developed by human subjects against selected *Mycobacterium tuberculosis* antigens were evaluated to assess the potential use of some of these antigens in a serodiagnostic test to discriminate between infected healthy subjects and TB patients.

A total number of 179 sera were analyzed: 52 sera were obtained from PPD-negative individuals, 38 sera were obtained from PPD-positive healthy subjects (BCG-immunized and *M. tuberculosis*-infected persons), and 111 sera were collected from patients with active TB. Both controls and samples from TB patients were obtained from the Clinic of Infectious Diseases, Institute of Tisiology, University of Sassari, and the Institute of Tisiology A.S.L. no. 3 of Nuoro, Italy.

Recombinant histidine-tagged proteins were purified by nickel chromatography as indicated previously (6). The recombinant methylated HBHA was purified from the *Mycobacterium smegmatis* pMV3-38 strain as described in Delogu et al. (5).

Specific antibodies against the selected purified *M. tuberculosis* antigens were detected by enzyme-linked immunosorbent assay (ELISA) following standard procedures. Briefly, ELISA plates were coated with 0.5 μg of recombinant protein in 0.1 ml of 0.1 M carbonate buffer (pH 9.5) overnight at 4°C. Plates were blocked with 1% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline (PBS) (pH 7.2) for 1 h. Sera from human patients and controls were diluted 1:20 in PBS containing 0.05% Tween 20 (PBS-T), and plates were incubated for 2 h at room temperature. Plates were washed five times with PBS-T, and whole anti-human immunoglobulin G antibody (Sigma-Aldrich) was added at a 1:1,000 dilution to PBS-T. After 1 h of incubation, plates were washed five times in PBS-T, and 0.1 ml of *p*-nitrophenylphosphate (Sigma-Aldrich) was added to each well. Plates were incubated until the color developed and were read at 405 nm by using a VERSA Tunable Max microplate reader (Molecular Devices).

Standardization of the ELISAs performed on different days was obtained, including three to six positively reacting sera (optical density [OD] values against methylated HBHA ranging between 0.8 and 1.1) for each plate. OD values for each serum gave differences in the order of 5 to 10% between the

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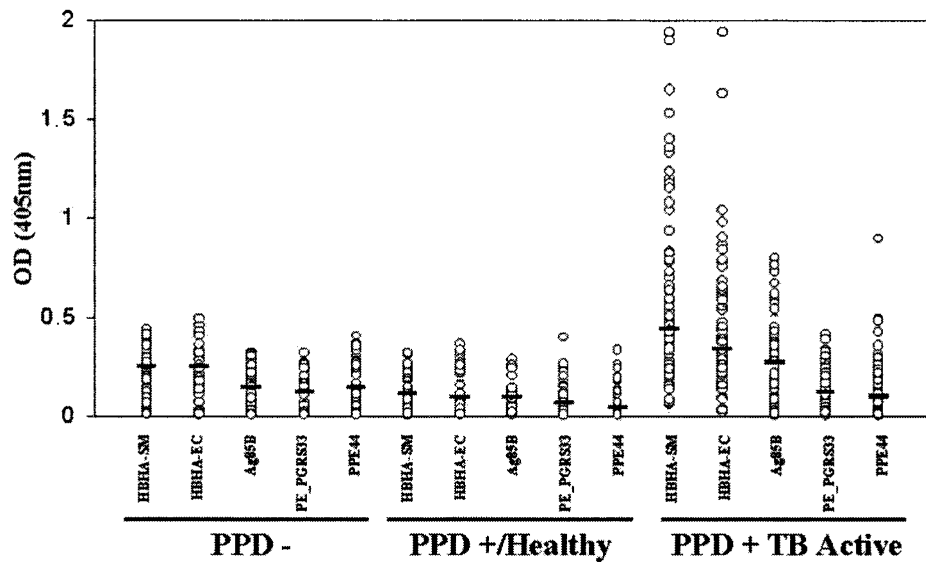

FIG. 1. Evaluation of serum samples from healthy donors (PPD−), healthy PPD-positive patients (PPD +/Healthy), and patients with TB (PPD + TB Active). Data are presented as values of OD_{405} observed following ELISA, as described in the text. Data from a representative experiment out of three are shown. The median value for each group is indicated by a dark solid horizontal line. HBHA-SM, HBHA purified from *M. smegmatis* pMV3-38 (5); HBHA-EC, HBHA purified from *E. coli* BL21(DE3)pLysS pGD51 (6).

different experiments, and results of one representative experiment among five to seven ELISAs are shown.

Sera collected from patients suffering from pulmonary TB were tested by ELISA for the presence of specific antibodies directed against four *M. tuberculosis* antigens: Ag85B, PPE44, PE_PGRS33, and HBHA. Pulmonary TB was demonstrated by radiological analysis and other clinical tests. Sera from noninfected PPD-negative patients were assayed and used as negative controls. Moreover, sera from PPD-positive healthy subjects were tested to assess the ability of the assay to discriminate healthy subjects from people suffering from TB.

Four recombinant *M. tuberculosis* proteins were used in these studies. The Ag85B antigen was selected because it is a highly immunogenic, actively secreted protein of *M. tuberculosis*. Usually, the host develops a strong cell-mediated immune (CMI) response against Ag85B, although specific antibodies have been detected (9). More than 100 proteins that belong to the PPE and PE_PGRS protein families have been found in the *M. tuberculosis* complex and a few other mycobacteria (2, 4). It has been demonstrated that the host mounts a strong CMI response against some of the PPE proteins (8). PE_PGRS proteins are highly conserved proteins that appear to be surface exposed and that may play a critical role in the host-microbe interactions (2). It has been shown that both in animal models and in human patients, a significant humoral response can be developed against these proteins, primarily targeting the repetitive PGRS domain (7, 14). HBHA is an *M. tuberculosis* adhesin involved in the dissemination of the bacilli from the site of primary infection (12). Masungi et al. have demonstrated that healthy *M. tuberculosis*-infected subjects develop a strong Th1-type immune response, while patients suffering from the active form of TB develop a strong humoral response (10). Since native HBHA is methylated in mycobacteria and methylation provides important immunological properties to the protein (15), a methylated form of HBHA obtained by *M. smegmatis* (5) was tested in these studies.

As shown in Fig. 1, the results of this study indicate that low levels of antibodies against the PPE and PE_PGRS proteins are detected in patients with active TB. In fact, the ranges of OD at 405 nm (OD_{405}) readings were very similar for the two groups of proteins, both for the control and infected groups, and the OD_{405} readings were always lower than 0.5. No statistically significant differences could be observed between the control group and the group with active TB ($P = 0.3301$; odds, 0.95) for sera reacting against these two proteins. Levels of specific antibodies against the Ag85B protein were very low for the control groups (PPD-negative and PPD-positive healthy subjects), and in only 11 out of the 111 TB patients were OD_{405} readings higher than 0.5, although a statistically significant difference between these groups could be observed ($P = 0.0187$; odds, 5.53). Antibody levels were slightly higher against the recombinant HBHA protein purified from *E. coli*. In this group, ELISA readings were low for the control groups and higher for the group of TB patients, and a statistically significant difference between these two groups was observed ($P = 0.0003$; odds, 13.18). Some of the TB patients showed levels of antibodies that were relatively high, and in 24 out of 111 patients, readings were higher than 0.5.

A statistically significant difference was observed between the control group and the group of patients with active TB when these sera reacted against methylated HBHA ($P = 0.00001$; odds, 28.23). As shown in Fig. 1, OD_{405} readings for sera collected from TB patients were higher when methylated HBHA was used with the ELISA. Forty-four out of 111 sera showed OD_{405} readings higher than 0.5, while the readings for the control groups were similar to those observed with the other antigens. Moreover, in the group of sera from TB patients, a comparative analysis of the reading values measured

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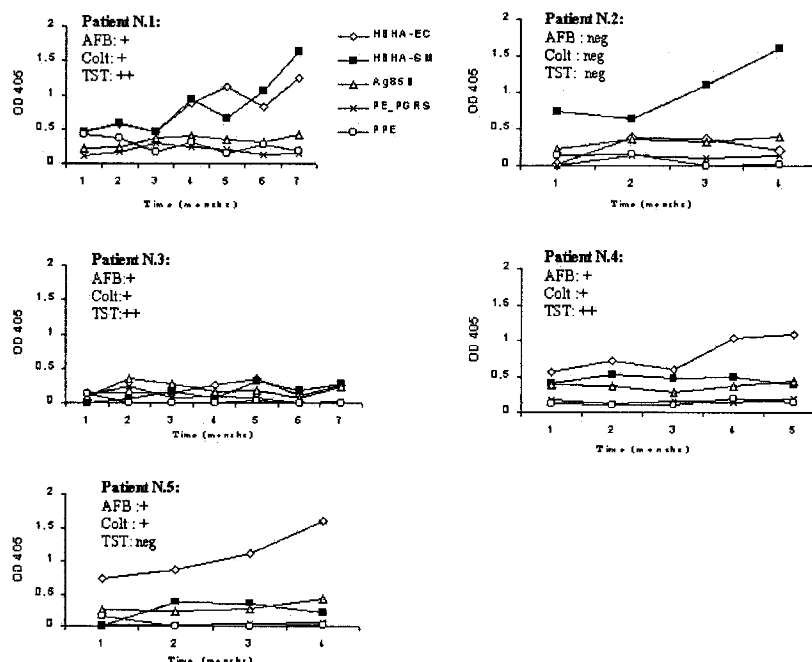

FIG. 2. Specific humoral responses developed by selected TB patients (no. 1 to no. 5 [N.1 to N.5]) over time against selected *M. tuberculosis* recombinant antigens. Abbreviations and symbols: HBHA-SM, HBHA purified from *M. smegmatis*; HBHA-EC, HBHA purified from *E. coli*; AFB, acid-fast bacillus staining; Colt, culture examination; +, positive; neg, negative; ++, induration of >10 mm.

with the different antigens indicated that reading values obtained with methylated HBHA were at least twice as high in 72 out of 111 sera than with the respective sera measured for PE_PGRS33 and in 68 out of 111 sera measured for PPE44. A similar comparative analysis carried out using the reading values obtained with the recombinant nonmethylated HBHA indicated that reading values were at least twice as high in only 55 out of 111 sera for the PE_PGRS33 group and in 50 out of 111 for the PPE44 group. Taken together, these data indicate that HBHA is a major target of the host humoral response during active TB and that good sensitivity and specificity of the ELISA can be obtained using the methylated form of the protein.

The prompt detection of TB patients in the early stages of infection warrants effective containment and therapy. To address this issue, sera from five TB patients were analyzed over a period of at least 4 months. Sera were collected at intervals of 1 month and assayed by ELISA using the selected *M. tuberculosis* antigens. As shown in Fig. 2, antibody titers against methylated HBHA were higher compared to those for the other antigens, beginning with the early stages of infection and increasing during therapy. In four out of five patients, a remarkable increase in antibody titers against HBHA was observed, though in three of these patients the antibodies could not recognize recombinant nonmethylated HBHA. Levels of antibodies against the other recombinant antigens remained low and did not significantly change during the time frame considered.

Interestingly, two of the patients with high antibody titers against methylated HBHA showed a negated tuberculin skin test (TST).

Overall, these results confirm that HBHA is differentially recognized by infected healthy subjects versus TB patients in serological assays (10) and that methylated HBHA may represent a good candidate for the development of new diagnostic tests aimed at identifying people suffering from the active form of the disease. The fact that in two of the patients that mounted a strong specific humoral response against methylated HBHA the TSTs were negative may suggest that such an HBHA-based serological test may be used in association with the TST in the diagnosis of TB or with more specific and sensitive T-cell-based assays (11). Further studies including a larger number of patients, the assessment of the specific HBHA-specific CMI response, and more relevant clinical and microbiological data are required in order to determine the usefulness and effectiveness of such HBHA-based diagnostic tests for TB.

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