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Anti-16-Kilodalton Mycobacterial Protein Immunoglobulin M Levels in Healthy but Purified Protein Derivative-Reactive Children Decrease after Chemoprophylaxis▽

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Serum responses against *Mycobacterium tuberculosis* HSP16 were determined for children with tuberculosis (TB) and for healthy purified protein derivative (PPD)-positive and PPD-negative children. Immunoglobulin G (IgG) and IgM responses were higher for TB patients than for other groups. After chemotherapy, IgM and IgG responses decreased for TB patients and PPD-positive subjects. Monitoring of anti-*M. tuberculosis* HSP16 responses could assist in the management of pediatric TB.

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the most relevant worldwide health problems, resulting in at least 1.5 million deaths each year (10, 11). Although pediatric cases represent a small percentage of all TB cases, infected children are a source of transmission of mycobacteria to adults (4, 5). In recent years, researchers' attempts have been directed toward the characterization of mycobacterial antigens that interact with the host humoral immune system (3, 6, 9). However, knowledge about humoral immune responses in children with TB is limited, and therefore, we investigated serum immunoglobulin G (IgG) and IgM responses to *M. tuberculosis* HSP16, a protein that is part of the latency operon (DosR) and is associated with dormant bacilli (7, 13).

Children who had not been vaccinated with *Mycobacterium bovis* BCG and who were from the same geographical area of Sicily and from similar socioeconomic backgrounds were recruited. Diagnosis of TB was established by the presence of clinical symptoms of TB, by chest radiography, by a positive result on the tuberculin (purified protein derivative [PPD]) skin test, and by symptomatic improvement after chemotherapy. All TB patients ($n = 45$; age range, 1 to 13 years; average age ± standard deviation [SD], 5.61 ± 3.24 years) included in this study had positive PPD skin tests. TB patients were treated with a commonly used mixture of antitubercular drugs containing isoniazid, rifampin, and pyrazinamide. Healthy children included in this study were not household contacts of known TB cases and were divided into two groups: PPD reactive (PPD^+) ($n = 60$; age range, 1 to 14 years; average age ± SD, 7.1 ± 4.1 years) and non-PPD reactive (PPD^-) ($n = 17$; age range, 3 to 14 years; average age ± SD, 7.2 ± 3.5 years). PPD^+ subjects were treated with isoniazid only and followed the same schedule used for TB patients.

Enzyme-linked immunosorbent assay plates (MaxiSorp; Nunc, Copenhagen, Denmark) were coated overnight at 37°C with 50 μl/well of 1-μg/ml recombinant HSP16 (a gift from J. Ivanyi) in carbonate buffer (0.1 M sodium bicarbonate in distilled water [pH 8.2]). Plates were blocked with phosphate-buffered saline (PBS)–10% fetal calf serum for 1 h at 37°C. Plates were incubated for 90 min at 37°C with 50 μl of serum samples diluted 1:50 in PBS. After four washes with PBS-Tween, 50 μl of anti-human IgG or IgM (both peroxidase conjugated; Sigma-Aldrich, Milan, Italy) was added to each well, and the mixture was incubated for 2 h at 37°C. The plates were washed six times with PBS-Tween and then colorimetrically developed with *o*-phenylenediamine (Sigma-Aldrich) in 0.1 M citrate phosphate buffer in the presence of H_2O_2. Plates were read as the optical density at 492 nm (OD_{492}) with an enzyme-linked immunosorbent assay multiwell reader (Sigma-Aldrich). After subtraction of background values, represented by the OD detected in wells without antigen, samples showing an OD of >0.150 were considered positive. Differences among group means were evaluated by the Mann-Whitney test. *P* values of <0.05 were considered significant.

To assess whether determination of HSP16-specific antibody responses could be useful for monitoring the efficacy of chemotherapy, we measured HSP16-specific IgG and IgM levels in sera from patients and healthy controls (PPD^+) before and after therapy. The results are presented as percentages of anti-HSP16 IgG- and IgM-positive sera (Fig. 1) and as mean ODs before and after chemotherapy (Fig. 2). The percentages of HSP16-specific IgG and IgM responders decreased after chemotherapy both for TB patients (from 73.3 to 37.7% for IgG; from 73.3 to 20% for IgM) and for

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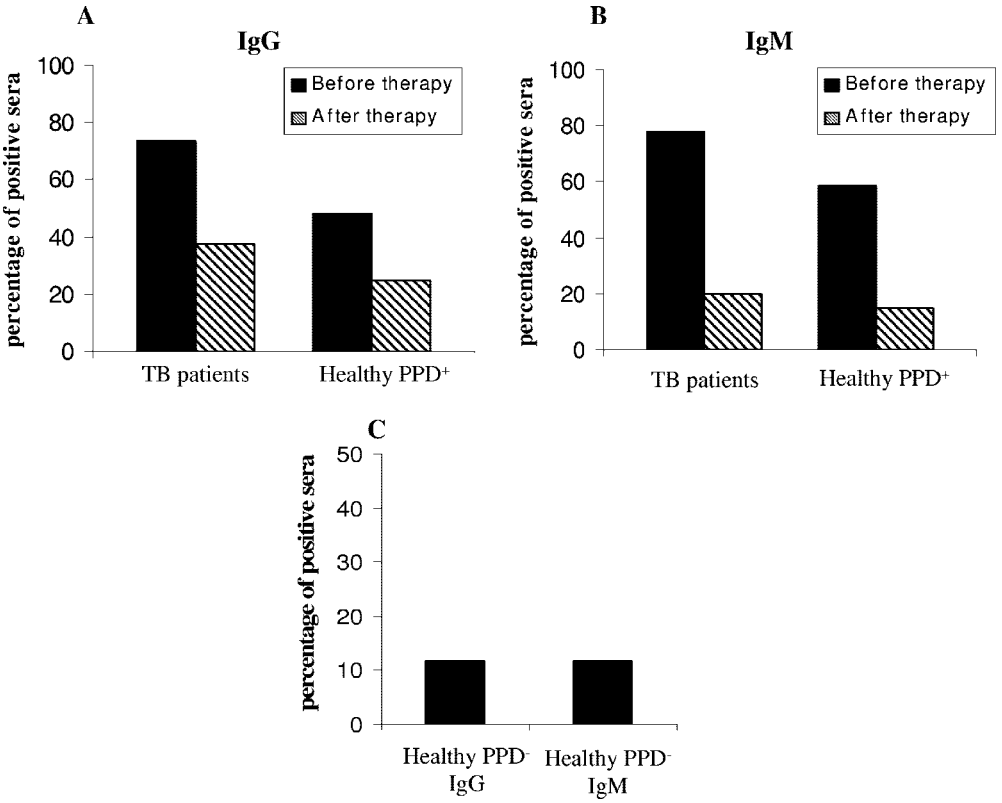

FIG. 1. Frequencies of anti-HSP16 IgG or IgM responses before and after chemotherapy. Shown are percentages of sera with IgG and IgM responses to HSP16 for TB patients ($n = 45$) (A) and for healthy PPD$^+$ ($n = 60$) (B) and PPD$^-$ ($n = 17$) (C) subjects. Frequencies of positive sera were obtained by dividing the number of positive sera for each group by the total number of sera tested for that group. Hatched bars, results obtained with sera after 4 months of therapy.

healthy PPD$^+$ subjects (from 48.3 to 25% for IgG; from 58.33 to 15% for IgM) (Fig. 1A and B, respectively). Percentages of sera positive for HSP16-specific IgG or IgM were low for PPD$^-$ patients (Fig. 1C).

Further, anti-*M. tuberculosis* HSP16 IgG (Fig. 2A and B) and IgM (Fig. 2C and D) levels of TB patients and PPD$^+$ subjects were compared before and after therapy. Before therapy, mean responses were higher for TB patients (ODs, 0.330 ± 0.231 for IgG and 0.390 ± 0.266 for IgM) than for healthy PPD$^+$ (ODs, 0.167 ± 0.147 for IgG and 0.237 ± 0.213 for IgM) or PPD$^-$ (ODs, 0.109 ± 0.135 for IgG and 0.104 ± 0.162 for IgM) subjects. After chemotherapy, IgG and IgM levels decreased more in TB patients (ODs, 0.190 ± 0.174 and 0.120 ± 0.085, respectively) than in healthy PPD$^+$ individuals (ODs, 0.113 ± 0.078 and 0.094 ± 0.067, respectively) ($P < 0.05$ for all parameters).

We found that levels of IgG and IgM against the 16-kDa antigen in infected children and in healthy contacts decrease after therapy. The recognition of the 16-kDa antigen is probably due to *M. tuberculosis* infection, since it has been observed mainly for patients, as confirmed by a study of adults (12). This observation could be interpreted as due to activation of memory B-cell clones against environmental mycobacteria in patients but not in adult contacts exposed to previous therapy. Naïve B-cell clones in children exposed to mycobacteria could induce the secretion of antibodies also in healthy contacts not previously treated with drugs. The response to the 16-kDa antigen could be associated with ESAT-6; in fact, antibodies against HSP16 and ESAT-6 could be detected during tubercular infection (1). The fall in 16-kDa-antigen responses after therapy has also been documented for adults (2). It has been reported that anti-16-kDa antibody levels in children could be elevated in response to infections, even without clinically apparent TB (8). These data are in agreement with our findings that IgG and IgM against the 16-kDa antigen were also detected in healthy PPD$^+$ subjects. In particular, the novelty of our finding lies in the decrease in the level of the IgM response in healthy PPD$^+$ children after prophylaxis. These data confirm that healthy contacts recognize the 16-kDa protein, as previously reported (2), and also that IgM could be useful for detecting the efficacy of antitubercular prophylaxis. The decrease in anti-16-kDa-antigen IgM levels after therapy in healthy PPD$^+$ contacts could be due to the inefficacy of therapy rather than a primary antibody response against a marker of latent infection. Our interpretation of these data could mean that after therapy, there is a percentage of children who still remain susceptible to mycobacterial infection. On the other hand, the persistence of IgG against the 16-kDa antigen could be a marker of serological memory that could protect individuals against reinfection.

In conclusion, the dynamics of anti-HSP16 IgG and IgM responses in children during chemotherapy could be used alongside other diagnostic and clinical criteria to monitor

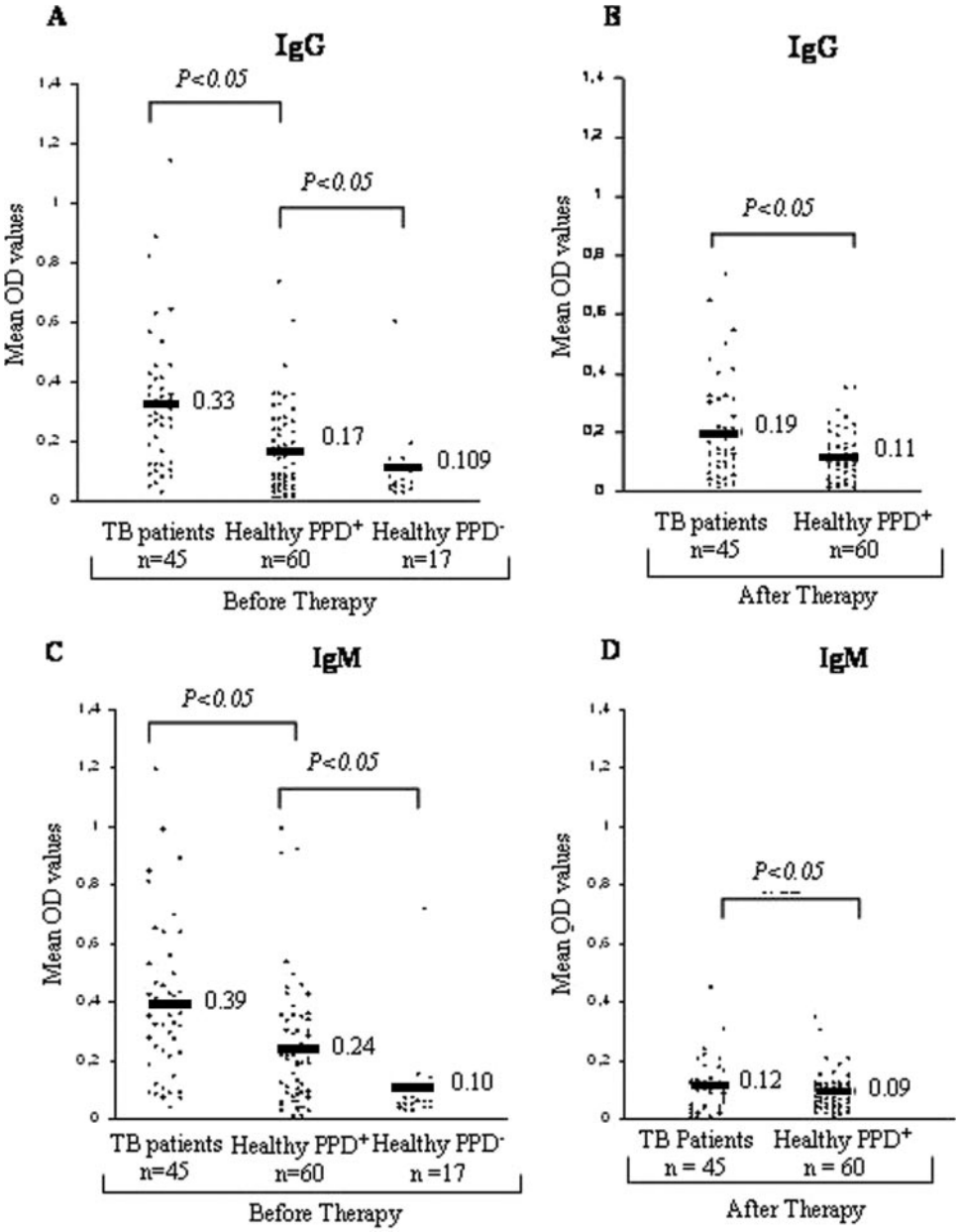

FIG. 2. Comparison of mean ODs of anti-HSP16 IgG before and after chemotherapy. (A and B) Mean ODs for anti-HSP16 IgG were analyzed before (A) and after (B) chemotherapy. For panel A, statistical comparisons were performed between data obtained with sera from TB patients and those from healthy PPD^+ subjects ($P < 0.05$) and between data for healthy PPD^+ versus PPD^- subjects ($P < 0.05$). For panel B, data from TB patients were compared with data from healthy PPD^+ subjects ($P < 0.05$). (C and D) Mean ODs for anti-HSP16 IgM were analyzed before (C) and after (D) chemotherapy. For panel C, statistical comparisons were performed between data for TB patients versus healthy PPD^+ subjects ($P < 0.05$) and between data for healthy PPD^+ versus PPD^- subjects ($P < 0.05$). For panel D, data obtained from TB patients were compared with data for healthy PPD^+ subjects ($P < 0.05$).

the efficacy of antitubercular drugs, not only for TB patients but also for healthy contacts, who are a potential reservoir for the dissemination of tubercle bacilli.

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