Antibodies to *Mycobacterium paratuberculosis* in Patients with Crohn's Disease

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IgG antibodies against *Mycobacterium paratuberculosis* protoplasmic antigen were looked for by an enzyme immunosorbent assay in patients with Crohn's disease, ulcerative colitis, active pulmonary tuberculosis, past pulmonary tuberculosis, and in healthy controls. Serum reactivity for these antibodies was not correlated to PPD skin test positivity without history of mycobacterial disease. A cutoff based on the mean absorbance value of a pool of healthy blood donors was chosen. Positive values were found in 12/24 (50%) patients with active pulmonary tuberculosis and 2/10 (20%) skin-test-positive subjects with past pulmonary tuberculosis as expected, because antigenic sharing is common among different mycobacteria. The control group of blood donors showed borderline positivities in 3/149 cases (2.01%). Positive values were found in 4/108 (3.70%) patients with Crohn's disease and 2/40 (5%) with ulcerative colitis. We conclude that our data do not support a causal relationship between the *M. paratuberculosis* and Crohn's disease, but occasional patients with inflammatory bowel diseases show unexpected positivities for these mycobacterial antibodies.

**KEY WORDS:** Crohn's disease; *Mycobacterium paratuberculosis*; inflammatory bowel disease; enzyme-linked immunosorbent assay; serodiagnosis; mycobacterial diseases.

In 1984 Chiodini and coworkers described an unclassified *Mycobacterium* species cultured from tissues of two patients with Crohn's disease and pathogenic for mice and goats (1, 2). Some other reports about positive cultures for mycobacteria in patients with Crohn's disease have then been published (3–6). Recent studies (7, 8) have demonstrated, by means of genetic analysis, that the microorganism isolated by Chiodini and coworkers is identical to the *Mycobacterium paratuberculosis*, the causal agent of Johne's disease, a chronic granulomatous enteritis of the ruminants (9).

Additional indirect evidence of exposure to mycobacterial agents was claimed by Thayer et al, who developed an enzyme-linked immunoassay coupled to the protoplasmic antigen of *Mycobacterium paratuberculosis*: they described a significant raised level of antibodies against protoplasmic antigen of *M. paratuberculosis* (PPA) in serum of patients with Crohn's disease in comparison to control patients (10). These serologic results were never replicated by other similar studies (11–14).

Our paper reports the results of a study carried out by enzyme immunoassay in order to evaluate the levels of specific IgG against PPA in patients with Crohn's disease in comparison to patients with...
Ulcerative colitis, healthy subjects, and subjects with previous or current pulmonary infection from *M. tuberculosis*.

**MATERIALS AND METHODS**

**Serum Specimens.** Sera were obtained from 108 patients with Crohn’s disease (CD), 24 patients with active pulmonary tuberculosis, 10 PPD skin-test-negative healthy subjects, 23 PPD skin-test-positive subjects subdivided in three classes (five without history of past mycobacterial diseases, eight BCG vaccinated, ten with history of past pulmonary tuberculosis), 40 patients with ulcerative colitis (UC), and 149 blood donors. The patients with CD and UC were diagnosed by clinical, radiologic, endoscopic, and histologic criteria according to Clamp et al (15), Johnson and Roth (16), Pera et al (17), and Morson and Dawson (18) and were randomly selected for this study. The majority of patients with CD were outpatients with Crohn’s disease activity index <150 (19) and without steroid therapy.

Twenty-five of the 108 patients with CD were screened for skin test reactivity to PPD. The PPD skin test positivity and negativity were defined according to the diagnostic standards of the American Thoracic Society Committee (20).

In order to exclude anergic patients, every PPD-negative patient with Crohn’s disease was included only if positive for at least one of six different antigens (tetanus and diphtheria toxins, *Streptococcus*, *Proteus*, *Trichophyton*, *Candida*).

The patients with pulmonary tuberculosis presented a typical radiological picture and positive culture of *M. tuberculosis*. Sera of healthy subjects with positive or negative skin tests were obtained from outpatients. The blood donors were randomly selected and standardized for sex and age from a batch of sera obtained in five following days at a local blood bank. The mean ages of CD patients, UC patients, and blood donors were, respectively, 38.25 (range 18-75), 47.9 (21-79), and 38.7 (18-64) years; the male-female ratios were, respectively, 38.25:1, 38.9:1, and 38.7:1. The majority of patients with CD were outpatients with Crohn’s disease activity index <150 (19) and without steroid therapy.

Antigens. *Mycobacterium paratuberculosis* proteolytic antigen (USDA strain 18) was obtained from Allied Laboratories Inc., Ames, Iowa; this antigen is a blend of 18 major polypeptides and proteins obtained by means of disruption, centrifugation, dialysis, and filtration of mycobacterial cells (21).

*Mycobacterium tuberculosis* PPD was obtained from Istituto Sieroterapico Berna, Como, Italy.

The skin test for tetanus and diphtheria toxins and *Streptococcus*, *Proteus*, *Trichophyton*, *Candida* antigens was performed by the Multitest IMC, Institut Merieux, Lyon, France.

**Enzyme-Linked Immunosorbent Assay (ELISA).** The indirect microplate ELISA was carried out as follows: 96-well polystyrene microplates were coated by passive adsorption with 200 µl of a PPA solution diluted to 1 µg/ml in carbonate buffer (pH 9.6) and incubated overnight at 4°C. The coated plates were washed five times with a 0.85% NaCl solution containing 0.1% Tween 20; the wells were then filled with 100 µl of serum diluted to 1:1000 in phosphate-buffered saline with 0.1% Tween 20 (PBS-Tween) and incubated overnight at 4°C. Each serum sample was tested in duplicate. After a second washing, 100 µl of horseradish peroxidase-conjugated anti-human IgG diluted to 1:1500 in PBS-Tween were added to each well and incubated for 60 min at 37°C. The wells were again washed and filled with 100 µl of orthophenylenediamine in phosphate-citrate buffer (pH 5.5) with H2O2 (0.15%). The enzymatic color reaction was stopped after 30 min at room temperature by adding 25 µl of 4 N H2SO4.

The results were determined photometrically by a microplate reader (MR 590, Dynatech) and expressed as extinction value at 492 nm (E492). The 0.00 value was determined for every plate by measuring the extinction value of a well not incubated with serum but otherwise fully processed. Positive (of a patient with pulmonary tuberculosis) and negative (of a healthy subject) control sera were included in every plate. Positive sera were defined those with an absorbance value greater than or equal to 0.450 E492; this cutoff was selected by adding three times the standard deviation to the arithmetic mean of the values detected in 149 healthy blood donors.

![Fig 1. Distribution of the ELISA PPA results in patients with Crohn’s disease and healthy controls selected for PPD skin test reactivity. E492: extinction at 492 nm; CD: Crohn’s disease; BCGv: BCG vaccinated.](image)

**RESULTS**

Healthy controls screened for PPD skin test reactivity, including BCG vaccinated subjects, showed no positive cases. No serum of patients with Crohn’s disease and PPD skin test positivity was positive; higher, but still negative, values were found in PPD-negative patients with Crohn’s disease (Figure 1).