Digestive Diseases and Sciences, Vol. 40, No. 12 (December 1995), pp. 2630–2634

Detection of Anti-Cord Factor Antibodies in Intestinal Tuberculosis for Its Differential Diagnosis from Crohn's Disease and Ulcerative Colitis

KAZUTOSHI KASHIMA, MD, PhD, SHIRO OKA, MD, PhD, AKIHIRO TABATA, MD, PhD, KATUNORI YASUDA, MD, PhD, ATUO KITANO, MD, PhD, KENZO KOBAYASHI, MD, PhD, and IKUYA YANO, MD, PhD

We have developed a diagnostic method for pulmonary tuberculosis by detecting antibody to cord factor using enzyme-linked immunosorbent assay (ELISA). This study was to evaluate the usefulness of our method for a diagnosis of intestinal tuberculosis, and especially its ability to differentiate this disease from other inflammatory bowel diseases. Antibodies of the immunoglobulin G class against cord factor (trehalose-6,6′-dimycolate) from 27 patients with intestinal tuberculosis, 16 patients with Crohn's disease (CD), and 27 patients with ulcerative colitis (UC) were tested by ELISA with cord factor purified from *Mycobacterium tuberculosis* H37Rv as the antigen. Twenty-three of the 27 patients with intestinal tuberculosis (85%) showed elevated values distinct from healthy controls. None of the patients with CD showed an elevation of antibody titers. Of the 27 patients with UC, 26 (96%) did not show any anti-cord factor antibody elevation. We conclude that this method is simple and results are reproducible. The results of our study justify undertaking the detection of anti-cord factor antibodies to diagnose intestinal tuberculosis.

KEY WORDS: Crohn's disease; cord factor; ELISA; intestinal tuberculosis; serodiagnosis; ulcerative colitis.

Cord factor (trehalose-6,6′-dimycolate; TDM), toxic glycolipid, is found widely among *Actinomycetes* such as *Mycobacterium, Nocardia,* and *Rhodococcus* (1–3) and may be related to mycobacterial virulence (1). Previously, Kato reported that an antibody against cord factor had been produced experimentally, but the antibody against cord factor was not found in the patients with pulmonary tuberculosis (4, 5). We recently found a high titer of antibodies against cord factor in the sera of patients infected with *M. tuberculosis* or nontuberculous mycobacteria (6). Measurement of IgG class antibody against cord factor in pulmonary tuberculosis is useful in a differential diagnosis of tuberculous or nontuberculous mycobacteriosis from other lung disease (6).

The accurate diagnosis of intestinal tuberculosis is essential, because it is infectious and its therapy is different from those of other inflammatory bowel diseases, such as Crohn's disease (CD) and ulcerative colitis (UC). The definite diagnosis of intestinal tuberculosis is still based on the detection of the acid-fast bacilli in secretions or biopsy specimens from patients, or on the histological findings of the granu-

Manuscript received March 2, 1995; revised manuscript received July 6, 1995; accepted August 18, 1995.

From the Department of Bacteriology and Third Department of Internal Medicine, Osaka City University, Medical School, Osaka, Japan.

Address for reprint requests: Dr. Kazutoshi Kashima, Department of Bacteriology, Osaka City University Medical School, 1-5-7, Asahimachi, Abeno-ku, Osaka 545, Japan.

0163-2116/95/1200-2630$07.50/0

loma formation in lesions. The results of tuberculin skin tests are not reliable in the final diagnosis of the active tuberculous diseases.

We have developed an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of pulmonary tuberculosis by the detection of an antibody to cord factor, a cell-wall glycolipid of *Mycobacterium tuberculosis*. Compared to other serodiagnostic tests for mycobacterial infection, the sensitivity and specificity of cord factor antibody were satisfactory (6).

The aim of this study was to evaluate the usefulness of our method for the diagnosis of intestinal tuberculosis, especially its ability to differentiate this disease from other inflammatory bowel diseases.

MATERIALS AND METHODS

Bacteria. Packed cells of *M. tuberculosis* H37Rv were used as sources of cord factor antigen.

Isolation and Purification of Cord Factor. Heat-killed packed bacteria were suspended in a 2:1 (v/v) mixture of chloroform and methanol and disrupted by ultrasonic treatment to extract the crude lipids. The chloroform layer was evaporated to dryness, and the residue was dissolved in a small volume of the same chloroform–methanol mixture. The crude lipid extracts were separated by thin-layer chromatography (TLC) on a silica gel plate (Uniplate, Analtech, Newark, Delaware) with a mixture of chloroform, methanol, acetone, and acetic acid (90:10:6:1, by vol) as the solvent. The band of cord factor was visualized with iodine vapor and recovered from the TLC plates with a mixture of chloroform–methanol (3:1, v/v). The purification was repeated until a single spot was obtained on the TLC plate.

Subjects. For this study, serum samples from 27 patients with intestinal tuberculosis, 16 patients with CD, and 27 patients with UC, and 100 healthy control were tested. Patients were between 18 and 70 years of age. There were 25 men and 45 women. We chose 10 intestinal tuberculosis patients in whom tuberculous bacilli were identified by cultivation of stool samples and 17 intestinal tuberculosis patients in whom tuberculous bacilli were not identified. In these groups, pulmonary tuberculosis lesions were not clearly detected by chest x-ray films. After antituberculous chemotherapy using streptomycin, isoniazid, and rifampicin for six months, ulcerative changes in these 27 patients were improved dramatically to scar formation, and we did not recognize recurrence in any cases more than 1 year after stopping medication.

The diagnoses of 16 patients with CD and 27 patients with UC were established on the basis of all available medical documentation including clinical, endoscopic, and pathological information. The 17 patients with CD included five with ileal type, nine with ileocolic type, and three with colic type disease. The 27 patients with UC included seven with rectal type, eight with left colic type, and 12 with total colic type disease. Every patient with CD or UC was diagnosed to be in the active stage at the time of blood sampling.

ELISA. ELISA was done on polystyrene microtiter plates (Falcon 3915, Becton Dickinson, Lincoln Park, New Jersey). The purified cord factor antigen was dissolved in *n*-hexane at a concentration of 0.1 mg/ml; 25 μl of the sample was placed in each well, and the plates were left to dry at room temperature. Then, 150 μl of phosphate-buffered saline solution (PBS-T) containing 0.05% Tween-20 was added to each well and removed by aspiration 1 hr after incubation at room temperature. After washing with PBS-T three times, serum diluted 1:160 (50 μl) was added to each well and the plate was incubated for 1 hr at room temperature. After washing with PBS-T three times, goat anti-human IgG or IgM labeled with peroxidase (Cappel Research Products, Durham, North Carolina) diluted 1:500 in PBS-T was added to each well as the second antibody. After incubation for 1 hr at room temperature and washing with PBS-T three times, the substrate, *o*-phenylenediamine (1 mg/ml) in 0.1 M citric acid, 0.2 M Na_2HPO_4 12 H_2O, and 0.04% H_2O_2 was added. The reaction was stopped with 6 N HCl, and the absorption was read in a microplate reader (MTP-100, Corona Electric, Ibaragi, Japan) at 492–630 nm.

Data Analysis. The test results were expressed as the absorption difference (difference of absorption value of the test serum from the same serum in wells without coated antigen). The data were analyzed by variance to Fisher's method as the *post-hoc* test.

RESULTS

In preliminary studies, we tested several dilutions of patients' sera and different amounts of antigen. The optimum serum dilution was estimated to be 1:160, and the optimum amount of antigen to be 2.5 μg/well. Therefore, the tests described below were done under these conditions.

Comparing IgG and IgM antibody titers against cord factor, the detection of IgG antibody was satisfactory, while IgM antibody titers of patients with intestinal tuberculosis were relatively lower than IgG, and some cases with CD or UC gave higher titers than healthy controls (data not shown). Based on the distribution of absorption values found in preliminary assays, the cutoff value was set at an optical density (OD) of 0.25; values equal to or larger than the cutoff value were taken to be positive.

The results of ELISA showed a bimodal distribution of anti-cord factor antibody titers for patients with intestinal tuberculosis and control groups, including patients with CD or UC (Figure 1). The mean OD of the patients with intestinal tuberculosis, in whom tuberculous bacilli were cultivated, was 0.446 (range 0.018–1.322). That of intestinal tuberculosis in which tuberculous bacilli were not detected was 0.368 (0.032–0.727). The mean OD of CD patients was 0.095 (0.012–0.186) and that in UC patients was 0.119

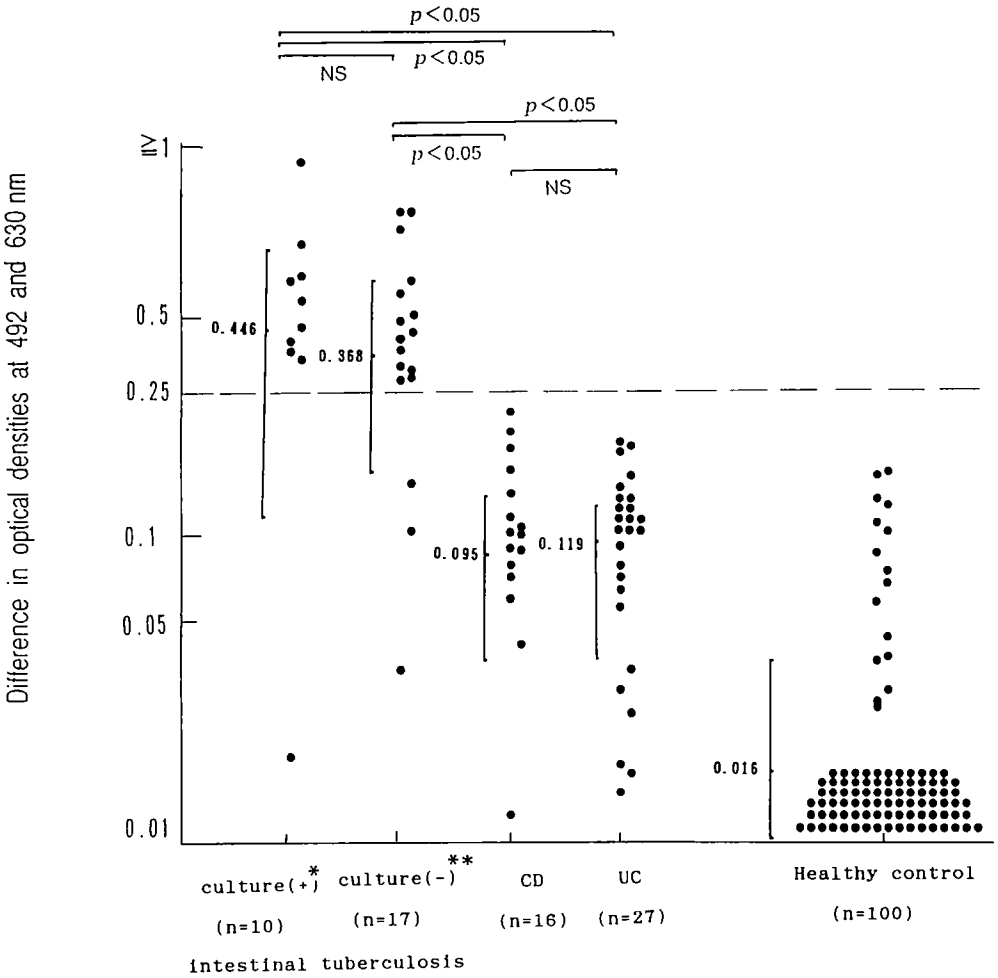

Fig 1. Anti-cord factor antibody titers in patients with intestinal tuberculosis, CD, and UC and in healthy controls. Numbers indicate mean OD and bars express standard deviations. * *M. tuberculosis* was identified in stool culture. ** *M. tuberculosis* was not identified in stool culture.

(0.023–0.141). On the other hand, OD of all healthy-controls was below 0.25 and mean OD was 0.016. The distribution of the results with a 0.25 cutoff point is shown in Table 1.

DISCUSSION

Several reports have been already published about the serodiagnosis of mycobacterial infections, in which polypeptides, polysaccharides, phospholipids, and components of the cell-wall skeleton including peptidoglycan arabinogalactan complex were used as antigens (7–12). For the detection of antibody, immunoprecipitation, agglutination, and immunodiffusion also, were utilized. However, the specificity of the antigens is low, and it is difficult to purify antigens for use in clinical tests. Therefore, a practical serodiagnosis has not been established (8, 9). Phenolglycolipid antigens (13, 14) and peptidoglycolipids (15, 16) have

TABLE 1. RESULTS OF ELISA IN PATIENTS WITH INTESTINAL TUBERCULOSIS, CD, AND UC*

Disease	*No. Positive/No. Tested*		*No. Negative/No. Tested*	
Intestinal TB				
Culture +	9/10 (90%)	23/27 (85%)	1/10 (10%)	4/27 (15%)
Culture −	14/17 (82%)		3/17 (18%)	
CD	0/16 (0%)		16/16 (100%)	
UC	1/27 (4%)		26/27 (96%)	

*Cutoff, OD 0.25.

been found in several mycobacterial species. These antigens are useful for the serodiagnosis and identification of certain species or serotypes of mycobacteria, although the structure of these antigens has not been fully established. Cord factor can be purified from *Mycobacterium, Nocardia,* and some related actinomycetes. This study showed that an ELISA with cord factor as antigen was useful for the rapid different diagnosis between intestinal tuberculosis and two other inflammatory bowel diseases.

In our previous study (6), in 99 infected patients (88 with pulmonary tuberculosis and 11 with atypical mycobacteriosis), 83 patients showed positive results (83.8%) by ELISA using cord factor as antigen when the cutoff value was set at an optical density of 0.1. Fifty-three patients in these 99 patients were culture-positive for mycobacteria in the sputum. In these 53 patients, 11 had been diagnosed as having atypical mycobacterial infection because of negative results in the niacin test of the cultivated bacilli, and all 11 patients had positive results by the ELISA. The mean OD of the patients with atypical mycobacteriosis was 1.050 (0.303–2.231). The sensitivity of atypical mycobacteriosis was 100%. These results suggested that anti-cord factor IgG antibody is produced commonly in mycobacterial infection. In this study, 16 patients with CD showed negative results by the ELISA. This suggests that mycobacterial infection is not involved in the etiology of CD, consistent with the results reported by Kobayashi et al (17, 18). Kobayashi et al tested sera of patients with active Crohn's disease and several control groups in an ELISA for reactivity with two mycobacterial antigens: lipoarabinomannan, a highly immunogenic somatic lipopolysaccharide present in the cell walls of all species of the *Mycobacterium* genus, and a protoplasmic antigenic preparation from *M.* sp. strain linda. They found no significant elevation in IgA, IgG, or IgM antibody levels to these two antigen preparations in the Crohn's disease patients.

IgG antibody against cord factor was detected in serum samples from patients with intestinal tuberculosis with a sensitivity of 85%, but was not encountered in CD patients (specificity 100%) nor UC patients (specificity 96%). The false negative rate for the diagnosis of intestinal tuberculosis was 15%, and the false positive in UC and CD patients was 21%. One patient with UC gave a positive result in the ELISA. This patient was a 33-year-old woman who had developed UC when she was 16 years old. The high OD may have been caused by subclinical mycobacterial infection.

Intestinal tuberculosis is almost entirely curable if diagnosis is made early enough and appropriate treatment is instituted. This report indicates that detection of anti-cord factor antibody in the sera of patients is useful for rapid diagnosis of intestinal tuberculosis and also helpful for differential diagnosis between intestinal tuberculosis and other inflammatory bowel disease.

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