Effect of intestinal resection on serum antibodies to the mycobacterial 45/48 kilodalton doublet antigen in Crohn’s disease

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Abstract
Interest in the role of mycobacterial infection in Crohn’s disease has been revived by the cultural detection of *Mycobacterium paratuberculosis* in patients with Crohn’s disease. This hypothesis was examined serologically using assays with high specificity for Crohn’s disease. The effect of intestinal resection on serum antibodies specific for Crohn’s disease was investigated with an immunoblot assay and an enzyme linked immunosorbent assay using the 45/48 kilodalton doublet antigen of *Mycobacterium tuberculosi*s. Antibodies were detected in 64-79% of patients with Crohn’s disease (n=17), 10% of patients with ulcerative colitis (n=10), 5% of patients with carcinoma of the colon (n=20), and none of 10 healthy subjects with the immunoblot assay. Statistical comparison of the Crohn’s disease patients with each control group resulted in p=0.0000236. Immunoglobulin G was essentially unchanged 75 days (mean) after surgery. After more than 180 days, however, the antibody response was reduced in all of five patients studied, and was no longer demonstrable in two of them (40%). Simultaneously, the Crohn’s disease activity index (CDAI) decreased. Both the high specificity of this assay for Crohn’s disease and the diminished antibody response after intestinal resection in parallel with decreased CDAI support a mycobacterial aetiology of Crohn’s disease.

(Keywords: Crohn’s disease, intestinal resection, *Mycobacterium paratuberculosis*.)

Many attempts have failed to detect increased antibodies directed to crude antigens of various disrupted mycobacteria in patients with Crohn’s disease.1–7 Similar results were obtained with purified common mycobacterial antigens like lipoarabinomannan8,9 or the membrane glycoprotein A6010 as well as purified antigens of *Mycobacterium paratuberculosis*.6,11 Increased immunoglobulin G (IgG) directed to the purified protein derivative of not only *M paratuberculosis*7 but also other mycobacteria have been found in Crohn’s disease.12 Increased IgA and IgM antibodies to the soluble antigens of *M tuberculosi*s in patients with Crohn’s disease were reported by Grange et al.13 Only Thayer et al.14 found an increased seroreactivity to a protoplasmic antigen of *M paratuberculosis* in patients with Crohn’s disease using an enzyme linked immunosorbent assay (ELISA). But Cho et al.15 failed to duplicate these results using the same antigen and an ELISA. Also Haga et al.16 were unable to reproduce the reported data and found that the antibody titres to *M paratuberculosis* of Crohn’s disease patients and controls did not differ in any immunoglobulin class.

Using an immunoblot technique with *M paratuberculosis* antigens separated by polyacrylamide gel electrophoresis, Whitehead17 found that identical antigen bands were recognised by the serum samples from the Crohn’s disease patients examined and cattle infected naturally with *M paratuberculosis*. From one of the patients with Crohn’s disease *M paratuberculosis* had been isolated. Finally, Das et al.18 showed with an immunoblot assay that IgG directed to the 45/48 kilodalton doublet antigen of *M tuberculosis* were present in 75% of the patients with Crohn’s disease compared with 20% in leprosy, 15% in tuberculosis, 20% in other bowel diseases, and 15% in systemic lupus erythematosus and rheumatoid arthritis.

Serum of these patients showed preferential reactivities to other mycobacterial antigens to which Crohn’s disease serum had no significant reaction. In the addition, the immunoreactive 45/48 kilodalton doublet antigen was also present in *M bovis* bacille Calmette-Guérin (BCG), *M avium*, and *M paratuberculosis*. The same investigators developed an ELISA to measure IgG antibodies to a gel purified 45/48 kilodalton doublet antigen of *M tuberculosis* in patients’ serum. They found increased IgG directed to this antigen pair in 65% of patients with Crohn’s disease compared with less than 8% in ulcerative colitis, less than 5% in diverticulitis, and less than 3% in carcinoma of the colon (unpublished findings). On the basis of both assays we investigated the influence of intestinal resection on the presence of these antibodies in patients with Crohn’s disease.

Methods
PATIENTS
Serum samples of 17 patients who underwent intestinal resection for Crohn’s disease (nine male, eight female; mean (SD) age 30 (12-3) years; range, 16–61) were obtained immediately or shortly before surgery (in two patients 22 days and in one patient 14 days before operation). Postoperatively 28 samples of all
control samples from 10 patients (five male, five female) with ulcerative colitis (mean (SD) age 32 (14-5) years), 20 patients with carcinoma of the colon (14 male, six female; 45 (15-3) years), and 10 healthy subjects (five male, five female; 38 (10-5) years) served as controls. Samples were stored at −20°C and thawed once under comparable conditions for further storage as aliquots.

The study was performed in accordance with the Helsinki Declaration II and was approved by the ethical committee of Heinrich-Heine-University Düsseldorf.

Figure 1: Homogeneity of 48 and 45 kilodalton (a and b respectively) gel purified protein antigen shown by SDS-gel electrophoresis followed by western blotting and amido black staining. The mixture of a and b (1:1) constituted 45/48 kilodalton doublet antigen for ELISA. m = molecular marker.

STUDY DESIGN

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE was performed in a discontinuous buffer system according to the method of Laemmli by using a 12% (w/v) separating gel containing 0-1% (w/v) SDS. Fifteen μg protein were applied per 810 mm² per run.

Immunoblot assay
The cytosol as well as the plasma membrane fraction of M tuberculosis were subjected to SDS-PAGE, and the separated bands were electrophoretically transferred to nitrocellulose using the method of Towbin et al. After washing, the nitrocellulose sheets were incubated with diluted patient's serum samples and then probed with horseradish peroxidase conjugated affinity purified secondary goat antihuman IgG (Cappel Laboratories, West Chester, PA, USA) as described. In each experiment low molecular weight standards (lysozyme 14 kilodalton, soybean trypsin inhibitor 22 kilodalton, ovalbumin 45 kilodalton, bovine serum albumin 66 kilodalton, phosphorylase B 93 kilodalton; Bio-Rad Laboratories, Richmond, CA, USA) were subjected to these procedures as described above and stained with amido black to facilitate the identification of the antigenic moieties of the 45/48 kilodalton doublet antigen reactive to serum. The immunoblot assays were qualitatively graded by comparison with the 66 kilodalton band by two of the authors independently with identical results. For verification a third blinded evaluation was performed by a third investigator.

Purification of subcellular fractions of M tuberculosis
The cytosol (Cyt) and the plasma membrane fraction (P90) of M tuberculosis strain RIVM 7114, Bilthoven, the Netherlands, were prepared according to the modified method of Das et al as described by Rambukkana et al.

Purification of the 45/48 kilodalton doublet antigen of M tuberculosis
A sonicated preparation of M tuberculosis was applied to a preparative SDS-PAGE by using a 12% (w/v) separating gel containing 0-1% (w/v) SDS. Slices, 2 mm, were cut from the gel in the area of 45 and 48 kilodalton. Bound proteins were eluted by either electroelution or diffusion in distilled water. The homogeneity of the antigen preparations was tested by SDS-PAGE followed by electrophoretic transfer to nitrocellulose, staining with amido black and also incubation with pool serum from patients with Crohn's disease, which were known to have antibody activities to the 45/48 kilodalton doublet antigen in a western blot assay (Fig 1). The eluted 45/48 kilodalton doublet antigen was extensively dialysed against phosphate buffered saline (PBS), pH 7-4, and stored at −20°C. This gel purified preparation was used for ELISA.

ELISA with the 45/48 kilodalton doublet antigen of M tuberculosis
ELISA serology was performed according to an optimum evaluated standard method
Effect of intestinal resection on serum antibodies to the mycobacterial 45/48 kilodalton doublet antigen in Crohn's disease

22
93
kd
taining

Figure 2: Immunoblot assay using the plasma membrane fraction (P90) and cytosol (Cyt) of M tuberculosis for detection of IgG directed to the 45/48 kilodalton doublet antigen in serum of patients with Crohn's disease. P90 means the plasma membrane fraction yielded by centrifugation at 90,000 g. P90 and Cyt apply to each of the four examples of Crohn's disease serum samples examined in duplicate. 1a + b: Two negative samples before and 613 days after surgery. 2a: Strong bands at 45/48 kilodalton before surgery (patient 2 in Table). 2b: Weaker bands 772 days after surgery (patient 2 in Table).

using the 45/48 kilodalton doublet antigen, which was SDS-PAGE purified from the whole sonicated preparation of broken organisms of M tuberculosis as already described.

The microdilution plates (Greiner, Denmark) were coated with a pre-evaluated optimal amount of 0.5 μg protein of the 45/48 kilodalton doublet antigen per well in 0.067 M PBS, pH 7–4, and incubated for two hours at 37°C. The plates were washed thoroughly with PBS containing 0.05% (v/v) TWEEN 20 (PBS-TWEEN 20); this wash was followed by further incubation with 2% (w/v) bovine serum albumin in PBS-TWEEN 20 for two hours and a subsequent washing step. Serial dilutions of human serum up to 1:1600 in 10% (v/v) normal goat serum and 0.1% (v/v) TWEEN 20 were added to achieve antibody binding by incubation for one hour at 37°C; this procedure was followed by thorough washing. The plates were further incubated for one hour at 37°C with pre-evaluated dilutions of horse-radish peroxidase conjugated affinity purified secondary goat anti-human IgG in PBS containing 10% (v/v) normal goat serum and 0.1% (v/v) TWEEN 20. After washing, the enzyme activities were shown with the substrate 3,3',5,5'-tetramethylbenzidine plus dimethyl sulfoxide and H2O2 by continuing the reaction for 15 minutes at 25°C. The colour development was stopped with 50 μl of 2 N H2SO4 and optical density was measured at 450 nm in a Bio-Rad ELISA reader coupled to a Macintosh computer. The end point titres of IgG antibody in the serum of patients and controls were determined using serial dilutions of the samples followed by extrapolating the titration curve to an optical density value equal to or greater than two times that for the reagent blanks. Comparing the titration curves of serum IgG antibody to 45/48 kilodalton doublet antigen in the patients and control serum, resulted in the maximum difference in optical densities in these two groups being seen in 1:400 dilution of serum.

The laboratory evaluations were carried out in the Section of Experimental Dermatopathology, Institute of Pathology of the University of Amsterdam.

STATISTICAL ANALYSIS

The CDAI before and after surgery was analysed with the paired Student's t test. The exact Fisher's test was applied for comparison of the results of the immunoblot assays of Crohn's disease patients with each control group.

Results

Western blot analysis of serum showed IgG directed to the 45/48 kilodalton doublet antigen of M tuberculosis in 11 of 17 patients with Crohn’s disease (64-7%) and one patient each with ulcerative colitis (10%) and carcinoma of the colon (5%). Figure 2 shows two negative serum samples (1a + b) and two samples with strong bands of the 45/48 kilodalton doublet antigen (2a + b) from patients with Crohn's disease. These antibodies were not detectable in 10 healthy subjects. Comparison of the results of Crohn's disease patients with each control group resulted in a statistical significance of p=0.0000236.

Forty five samples obtained from the 17 patients with Crohn's disease were investigated with the immunoblot assay as well as the ELISA. Both assays yielded positive results in 20 samples and negative results in 16, but different results in nine samples: five samples were reactive in the immunoblot assay but unreactive in the ELISA, four samples were negative in the immunoblot assay but gave a positive reaction in the ELISA. Altogether, both assays yielded identical results in 80%.

Within a mean period of 284 days after intestinal resection 28 samples were obtained from these 17 patients with Crohn's disease.

The CDAI decreased significantly from an average of 343 (range, 102–679) to 168 (range, 10–376; p<0.0005). The first follow up samples were obtained from all patients a mean of 75 days after surgery (range, 5–613 days) and showed no significant change in the immunoblot assay: of the 11 positive samples (10 preoperatively, one postoperatively) the bands of the 45/48 kilodalton doublet antigen remained unchanged in three patients, whereas the bands of the other eight samples showed changes in intensity compared with the 66 kilodalton band. In addition, 75% of all samples showed an identical antibody time course in the immunoblot assay and the ELISA. In one patient the antibodies were not detected before surgery and nine days afterwards; they became
Serology and disease activity in Crohn’s disease patients with serum IgG against the 45/48 kilodalton doublet antigen of M tuberculosis before and more than 180 days after resection of intestine

<table>
<thead>
<tr>
<th>Patient</th>
<th>Interval after resection of intestine (days)</th>
<th>CDAI*</th>
<th>Immunoblot assay†</th>
<th>ELISA‡</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>337 (+)</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>13</td>
<td>230 +</td>
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<td>48 –</td>
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<td>1994</td>
<td>310 –</td>
<td>–</td>
<td>–</td>
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<tr>
<td>2</td>
<td>0</td>
<td>102 ++</td>
<td>++</td>
<td>++</td>
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<tr>
<td></td>
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* CDAI=Crohn’s disease activity index. † Negative, (+) weak positive, + distinct positive, ++ strong positive, ++/+ intermediate values.

weakly positive in the immunoblot assay 209 days later, but were again no longer demonstrable after a further 76 days. The ELISA remained continuously negative. After a mean postoperative interval of 538 days (range, 200–1924 days) samples of nine of the patients with Crohn’s disease were obtained additionally, in these the CDAI was decreased from 338 (range, 102–679) to 144 (range, 8–540; p<0·05). Preoperatively five of these (56%) had shown IgG against the 45/48 kilodalton doublet antigen in the immunoblot assay (Table). In three of these five patients (60%) the bands became slightly but noticeably weaker in the immunoblot assay compared with the 66 kilodalton band 342 days (mean; range, 202–772 days) after surgery, the CDAI decreased from 386 to 55. In two of these five patients (40%), who had weakly positive bands preoperatively, the IgG were no longer demonstrable in the immunoblotting assay at 1924 days (and additionally at 1994 days), and at 201 days after bowel resection, respectively. In these five positive patients the change in titre in the ELISA paralleled the immunoblot in 83-3% (Table). Figure 3 shows the weak bands of the 45/48 kilodalton doublet antigen in patient 5 (Table). They were no longer demonstrable 201 days after further subtotal colectomy with retention of the sigmoid colon and rectum, at which time the CDAI had decreased from 502 to 135. The other patient (number 1 in Table) had a resection of the anastomosis after ileotransversostomy, 1924 days after that the CDAI had decreased from 337 to 48, however, 70 days later the CDAI increased again to 310 without a change in the immunoblot assay and the ELISA.

None of these five patients had any history of tuberculosis. Multiple x rays of the thorax performed during a period of 7-5 (5-0) years (mean (SD); range 0·05–10·7) showed no radiological signs of tuberculosis in four patients. Only one patient (number 4 in Table) showed scarred residues of a minor specific infection but had a negative skin test with tuberculin in autumn 1993.

The immunoblot assay showed no positive correlation with the immunosuppressive treatment, or with the duration, intestinal involvement as well as the activity of Crohn’s disease. The preoperatively positive serum samples that were tested again after more than 180 days after intestinal resection yielded, however, a positive correlation with the CDAI in the immunoblot assay.

Discussion
On the basis of an immunoblot assay and an ELISA, specific IgG directed to the 45/48 kilodalton doublet antigen of M tuberculosis were demonstrable in 64-7% and 58-8%, respectively, of patients with Crohn’s disease, significantly higher than in controls and in other intestinal diseases. These data confirm the results of Das et al,18 who showed that the immunoblot assay has a specificity of about 80–90% for patients with Crohn’s disease, whereas the sensitivity of 65% is rather low. The 45/48 kilodalton doublet antigen was purified from tubercle bacilli because M paratuberculosis was not available in sufficient quantity. Furthermore, the 45/48 kilodalton doublet antigen belongs to the family of...
mycobacterial immuno cross reactive antigenic components,22 which is also present in M paratuberculosis as shown by immunoblotting using serum of Crohn's patients (unpublished finding). In the case of an unclear histological diagnosis this assay may be useful for differentiation of ulcerative colitis from Crohn's disease as well as for follow up.

An immunoblot assay using the protoplasmic and surface antigen of M paratuberculosis, which was recently described by Tanaka et al 25 shows certain parallels to our results. A strong IgG response against a 45 kilodalton antigen from the M paratuberculosis protoplasmic antigen was found in 40% of the 10 Crohn's disease patients, and it was also weakly present in a further 20%. A weak response, however, was also given by four ulcerative colitis (40%) and one of the non-inflammatory bowel disease patients (10%).

Using the western blot technique Elsaghir et al 26 were also able to detect antibodies binding to the 38 kilodalton band of the extract of M paratuberculosis in 57% of Crohn's disease patients. In addition, antibody levels to the 24 and 18 kilodalton bands assessed by ELISA were significantly increased in 53% of the patients with Crohn's disease compared with controls. More recently Stainsby et al 7 failed to detect increased antibodies to crude antigens of various disrupted mycobacteria, which is in accordance with earlier studies.1-6 A distinct subset of 25% of patients with active Crohn's disease, however, had increased IgG concentrations compared with controls.

Sanderson et al 27 were able to detect DNA of M paratuberculosis in tissues in 65% of patients with Crohn's disease, but only in 4-3% of patients with ulcerative colitis and 12.9% of control patients. Their data obtained with a highly specific polymerase chain reaction supplement ideally with our serological results.

The purpose of our investigation was to evaluate the follow up of serum antibodies specific for Crohn's disease after the resection of diseased intestinal segments. Seventy five days after surgery the IgG were essentially unchanged in the immunoblot assay and the ELISA. After more than 180 days, however, an antibody decrease occurred in all five patients studied at that interval, and in 40% these antibodies were no longer demonstrable. In these there was a positive correlation between the specific antibodies and the CDAI. But there was one exception in a patient in whom the CDAI increased again 70 days later without the detection of specific antibodies (Table). Tuberculosis was not responsible for the immunoreactivity of these five patients. A non-specific decrease in titres of antibodies to the 45/48 kilodalton doublet antigen caused by an artefact, for example, freeze thawing or dilution of serum could be excluded by the comparison with the 66 kilodalton band.

In summary, on the basis of a mycobacterial doublet antigen, a serological assay with high specificity for Crohn's disease was developed. The antibody response was reduced 200 to 1994 days after the resection of diseased intestinal segments in Crohn's disease, and was no longer demonstrable in 40%. This supports a mycobacterial aetiology of Crohn's disease. A prognostic significance of this assay may be speculated, but can only be confirmed on the basis of more data and longer follow up. Of special interest would be the effect of appropriate antimycobacterial chemotherapy on this antibody response.

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8 Blaser MJ, Miller RA, Lacher J, Singleton JW. Patients with active Crohn's disease have elevated serum antibodies to antigens of seven enteric bacterial pathogens. Gastroenterology 1984; 87: 888-94.


