

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

G Kreuzpaintner, P K Das, A Stronkhorst, A W Slob, G Strohmeyer

## 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 tuberculosis*. Antibodies were detected in 64.7% 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.**

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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.<sup>1-7</sup> Similar results were obtained with purified common mycobacterial antigens like lipoarabinomannan<sup>8,9</sup> or the membrane glycoprotein A60<sup>10</sup> as well as purified antigens of *Mycobacterium paratuberculosis*.<sup>6,11</sup> Increased immunoglobulin G (IgG) directed to the purified protein derivative of not only *M paratuberculosis*<sup>7</sup> but also other mycobacteria have been found in Crohn's disease.<sup>12</sup> Increased IgA and IgM antibodies to the soluble antigens of *M tuberculosis* in patients with Crohn's disease were reported by Grange *et al*.<sup>13</sup> Only Thayer *et al*<sup>14</sup> found an

increased serological reactivity to a proto-plasmic antigen of *M paratuberculosis* in patients with Crohn's disease using an enzyme linked immunosorbent assay (ELISA). But Cho *et al*<sup>15</sup> failed to duplicate these results using the same antigen and an ELISA. Also Haga *et al*<sup>16</sup> 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, Whitehead<sup>17</sup> 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*<sup>18</sup> 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 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

Bernhard Nocht  
Institute for Tropical  
Medicine, Hamburg,  
Germany  
G Kreuzpaintner

Departments of  
Dermatopathology  
P K Das  
A W Slob

and Gastroenterology  
A Stronkhorst

Academic Medical  
Centre, University of  
Amsterdam,  
Amsterdam, the  
Netherlands

Department of  
Gastroenterology,  
Heinrich-Heine-  
University Düsseldorf,  
Düsseldorf, Germany  
G Strohmeyer

Correspondence to:  
Dr G Kreuzpaintner,  
Bernhard Nocht Institute for  
Tropical Medicine,  
Bernhard-Nocht-Str 74,  
20359 Hamburg, Germany.

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17 patients were acquired after 284 (508) days (mean (SD); range 5–1994 days), two each in five patients, three each in three patients. The diagnosis of Crohn's disease had been established by clinical criteria as well as x ray, endoscopy, and histological assessment. The average duration of disease was 10 (6.2) years (range 1.7–25.6). In two patients only the ileum or the colon were affected and in 13 patients the large and small bowel were affected. In five patients the terminal ileum and the right hemicolon, in three patients the terminal ileum and caecum (in one of these additionally the sigmoid colon), in two patients a part of the ileum or the colon without sigmoid colon and rectum (in one of these additionally the terminal ileum) were resected. The resection of a previous anastomosis (one jejunum and transverse colon, one ileum and transverse colon, one ileum and ascending colon with abdominoperineal proctectomy), the resection of an ileal loop, and the left hemicolon as well as a sigmoidectomy was performed. At the time of surgery eight patients received immunosuppressive treatment: seven with systemic adrenocortical corticosteroids (five, flucortolone 5–30 mg/day, two methylprednisolone 10–16 mg/day, in one of these additionally betamethasone enema (2.5 mg/day)), and one with azathioprine (50 mg/day). Up to 32 days (mean) after surgery five patients were treated with systemic adrenocortical corticosteroids: four with flucortolone 5–20 mg/day, one with methylprednisolone 30 mg/day. Only one patient received 10 and 20 mg flucortolone per day, respectively, 1924 and 1994 days after surgery. The disease activity was assessed by the Crohn's disease activity index (CDAI), a numerical index calculated from the number of liquid or very soft stools, abdominal pain, general well being, inflammatory complications, abdominal mass, body weight, packed cell volume, and intake of loperamide or opiates.<sup>19</sup>

#### CONTROLS

Serum 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.

#### 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<sup>20</sup> by using a 12% (w/v) separating gel

containing 0.1% (w/v) SDS. Fifteen µg protein were applied per 810 mm<sup>2</sup> 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.*<sup>21</sup> After washing, the nitrocellulose sheets were incubated with diluted patients' serum samples (1:100) and then probed with horseradish peroxidase conjugated affinity purified secondary goat antihuman IgG (Cappel Laboratories, West Chester, PA, USA) as described.<sup>22</sup> 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.*<sup>23</sup> as described by Rambukkana *et al.*<sup>24</sup>

##### 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<sup>22</sup>

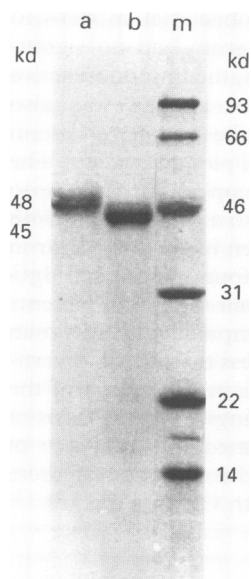


Figure 1: Homogeneity of 48 and 45 kilodalton (a and b respectively) gel purified protein antigen shown by SDS-gel electrophoresis followed by western blotting and amido black staining. The mixture of a and b (1:1) constituted 45/48 kilodalton doublet antigen for ELISA. m = molecular marker.

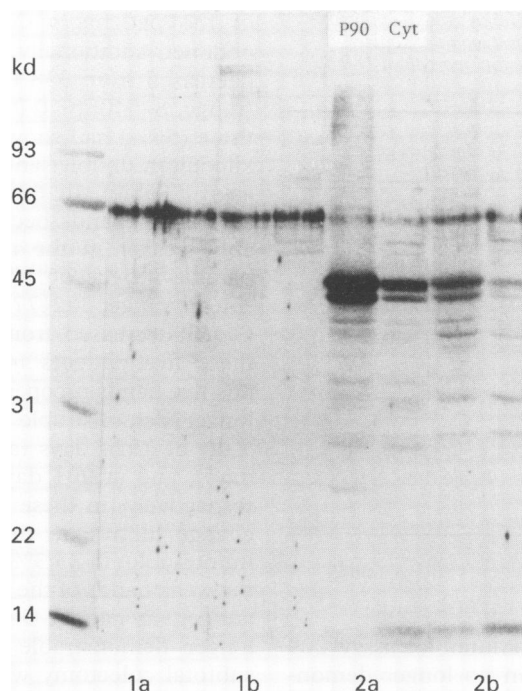


Figure 2: Immunoblot assay using the plasma membrane fraction (P90) and cytosol (Cyt) of *M tuberculosis* for detection of IgG directed to the 45/48 kilodalton doublet antigen in serum of patients with Crohn's disease. P90 means the plasma membrane fraction yielded by centrifugation at 90 000 g. P90 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 antigen 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 horseradish peroxidase conjugated affinity purified secondary goat antihuman 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 sulphoxide and H<sub>2</sub>O<sub>2</sub> by continuing the reaction for 15 minutes at 25°C. The colour development was stopped with 50 µl of 2 N H<sub>2</sub>SO<sub>4</sub>, 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*

Patient	Interval after resection of intestine (days)	CDAI*	Immunoblot assay†	ELISA‡
1	0	337	(+)	-
	13	230	+	+
	1924	48	-	-
2	1994	310	-	-
	0	102	++	++
	208	10	+++	+++
3	772	8	+++	+++
	0	679	++	++
	25	279	++	++
4	250	117	+++	+++
	276	123	+++	-
	0	378	+	(+)
5	20	119	+	+
	202	34	+/(+)	(+)
	0	502	(+)	+
	12	122	(+)	+
	201	135	-	+

\*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.*,<sup>18</sup> 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

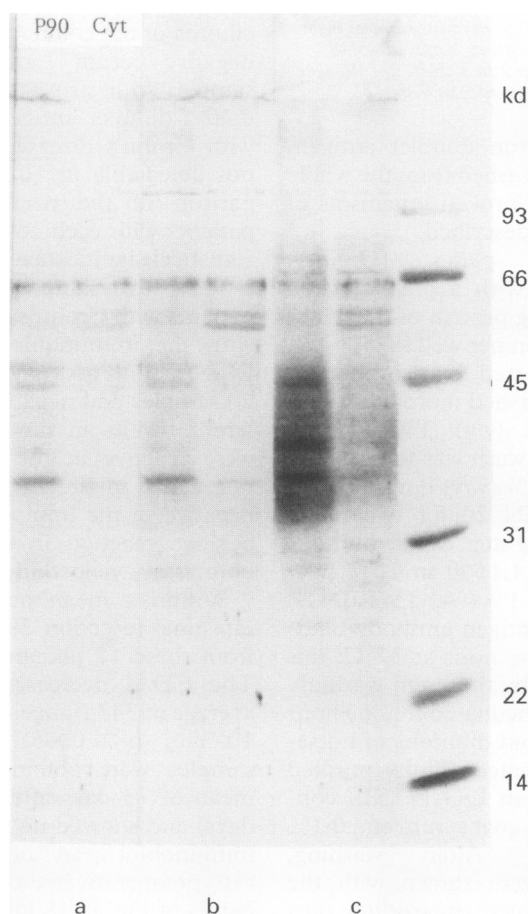


Figure 3: Immunoblot assay using the plasma membrane fraction (P90) and cytosol (Cyt) of *M tuberculosis* for detection of IgG directed to the 45/48 kilodalton doublet antigen in serum of patient 5 (Table) with Crohn's disease. a: Weak bands (P90) and very weak bands (Cyt) at 45/48 kilodalton before surgery. b: Weak bands in the plasma membrane fraction (P90) and no visible bands in the cytosol fraction (Cyt) at 45/48 kilodalton 12 days after surgery. c: No detection of IgG in both fractions at 45/48 kilodalton 201 days after surgery.

mycobacterial immuno cross reactive antigenic components,<sup>22</sup> 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*<sup>25</sup> 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 Elsaghier *et al*<sup>26</sup> 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*<sup>7</sup> failed to detect increased antibodies to crude antigens of various disrupted mycobacteria, which is in accordance with earlier studies.<sup>1-6</sup> A distinct subset of 25% of patients with active Crohn's disease, however, had increased IgG concentrations compared with controls.

Sanderson *et al*<sup>27</sup> 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 12.5% 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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- Morganroth J, Watson DW. Sensitivity to atypical mycobacterial antigens in patients with Crohn's disease. *Am J Dig Dis* 1970; 15: 653-7.
- Thayer WR Jr, Fixa B, Komarkova O, Charland C, Field CE. Skin test reactivity in inflammatory bowel disease in the United States and Czechoslovakia. *Dig Dis* 1978; 23: 337-40.
- Matthews N, Mayberry JF, Rhodes J, Neale L, Munro J, Wensinck F, *et al*. Agglutinins to bacteria in Crohn's disease. *Gut* 1980; 21: 376-80.
- Elliott PR, Lennard-Jones JE, Burnham WR, White S, Stanford JL. Further data on skin testing with mycobacterial antigens in inflammatory bowel disease [Letter]. *Lancet* 1980; ii: 483-4.
- Lefler HM, Marshall V, Lanspa SJ, Tremaine WJ. Detection of serum antibodies to Mycobacterium paratuberculosis from patients with Crohn's disease: a blinded study [Abstract]. *Gastroenterology* 1985; 88: 1470.
- Haagsma J, Mulder CJJ, Eger A, Tytgat GNJ. A study of antibodies to Mycobacterium paratuberculosis in inflammatory bowel disease. Preliminary results. In: MacDermott RP, ed. *Inflammatory bowel disease: current status and future approach*. Amsterdam: Elsevier Science, 1988: 539-41.
- Stainsby KJ, Lowes JR, Allan RN, Ibbotson JP. Antibodies to Mycobacterium paratuberculosis and nine species of environmental mycobacteria in Crohn's disease and control subjects. *Gut* 1993; 34: 371-4.
- 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.
- Kobayashi K, Brown WR, Brennan PJ, Blaser MJ. Serum antibodies to mycobacterial antigens in active Crohn's disease. *Gastroenterology* 1988; 94: 1404-11.
- McFadden JJ, Houdayer C. No evidence for antibodies to mycobacterial A60 antigen in Crohn's disease sera by enzyme-linked immunosorbent assay (ELISA). *J Med Microbiol* 1988; 25: 295-8.
- Funakoshi O. Mycobacterial aetiology of Crohn's disease. *Jpn J Gastroenterol* 1988; 85: 1340-6.
- Jiwa NM, Mulder CJJ, van den Berg FM, Tytgat GNJ, Haagsma J, Walboomers JMM. Elevated IgG to mycobacterial PPD's in Crohn's disease. In: MacDermott RP, ed. *Inflammatory bowel disease: current status and future approach*. Amsterdam: Elsevier Science, 1988: 543-6.
- Grange JM, Gibson J, Nassau E, Kardjito T. Enzyme-linked immunosorbent assay (ELISA): a study of antibodies to Mycobacterium tuberculosis in the IgG, IgA and IgM classes in tuberculosis, sarcoidosis and Crohn's disease. *Tubercle* 1980; 61: 145-52.
- Thayer WR Jr, Coudu JA, Chiodini RJ, van Kruijning HJ, Merkal RS. Possible role of mycobacteria in inflammatory bowel disease. II. Mycobacterial antibodies in Crohn's disease. *Dig Dis Sci* 1984; 29: 1080-5.
- Cho S-N, Brennan PJ, Yoshimura HH, Korelitz BI, Graham DY. Mycobacterial aetiology of Crohn's disease: serologic study using common mycobacterial antigens and a species-specific glycolipid antigen from Mycobacterium paratuberculosis. *Gut* 1986; 27: 1353-6.
- Haga Y, Funakoshi I, Nakajima H, Sano M, Murata A, Munakata A, *et al*. Antibodies to Mycobacterium paratuberculosis in sera of patients with Crohn's disease. *Digestive Organs Immunology* 1986; 17: 100-3.
- Whitehead JT. Comparison of Mycobacterium paratuberculosis with other Mycobacterium spp (including an isolate from a patient with Crohn's disease) by polyacrylamide gel electrophoresis and immunoblotting techniques. In: Thorel MF, Merkal RS, eds. *Proceedings of the second international Colloquium on Paratuberculosis, 1988 Sep 22-23, Maisons-Alfort, France*. Maisons-Alfort: Laboratoire Central de Recherches Vétérinaires, 1989: 254-64.
- Das PK, Blaauwgeers JLG, Slob AW, Yong SL, Rambukkana A. Mycobacterial antibody (myc-ab) response in relation to Crohn's disease (CD). *Gastroenterology* 1989; 96: A111.
- Best WR, Becketl JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. *Gastroenterology* 1976; 70: 439-44.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.

- 21 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350-4.
- 22 Das PK, Rambukkana A, Baas JG, Groothuis DG, Halperin M. Enzyme-linked immunosorbent assay for distinguishing serological responses of lepromatous and tuberculoid leprosy to the 29/33-kilodalton doublet and 64-kilodalton antigens of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1990; **28**: 379-82.
- 23 Das PK, Ibrahim AA, Binkhuysen F, Thi Hué P, Qui TH, Rangarajan R, *et al*. The use of subcellular components of BCG for studying host-mycobacterium interaction in relation to leprosy. *Ann Inst Pasteur Immunol* 1982; **133D**: 41-59.
- 24 Rambukkana A, Das PK, Chand A, Baas JG, Groothuis DG, Kolk AHJ. Subcellular distribution of monoclonal antibody defined epitopes on immunodominant *Mycobacterium tuberculosis* proteins in the 30-kDa region: identification and localization of 29/33-kDa doublet proteins on mycobacterial cell wall. *Scand J Immunol* 1991; **33**: 763-75.
- 25 Tanaka K, Wilks M, Coates PJ, Farthing MJG, Walker-Smith JA, Tabaqchali S. *Mycobacterium paratuberculosis* and Crohn's disease. *Gut* 1991; **32**: 43-5.
- 26 Elshagier A, Prantera C, Moreno C, Ivanyi J. Antibodies to *Mycobacterium paratuberculosis*-specific protein antigens in Crohn's disease. *Clin Exp Immunol* 1992; **90**: 503-8.
- 27 Sanderson JD, Moss MT, Tizard MLV, Hermon-Taylor J. *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. *Gut* 1992; **33**: 890-6.