

Mycobacterium paratuberculosis and Crohn's disease

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Abstract

The possible aetiological role of *Mycobacterium paratuberculosis* in Crohn's disease was investigated. The immunological response was studied using an enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunocytochemistry. The antibody response to two protoplasmic antigen preparations of *M paratuberculosis* in the sera of patients with inflammatory bowel disease was measured by ELISA. IgG and IgM antibodies to these antigens were measured in serum samples from 52 patients with Crohn's disease, 15 patients with ulcerative colitis, and 41 control patients without inflammatory bowel disease. Although there was wide variation in the concentrations of antibody detected, patients with Crohn's disease had concentrations that were not significantly different from those of the other two groups. In addition, mycobacterial antigens were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and the immune response to each antigen was then examined separately and assayed for IgG and IgM in 10 patients from each of the three groups. An indirect peroxidase test was also used to detect *M paratuberculosis* in sections of tissue from 18 patients with Crohn's disease and 10 with ulcerative colitis. The results were negative in all cases. This study does not support a role for *M paratuberculosis* in Crohn's disease.

Although the aetiology of Crohn's disease remains unknown, a variety of bacteria have been implicated.¹ Crohn's disease is a granulomatous ileitis histologically similar to that which occurs in tuberculosis,²⁻⁴ and this finding has prompted many investigators to attempt the culture of *Mycobacteria* from the tissue of patients with Crohn's disease.^{5,6} Granulomatous ileitis in ruminants, so called Johne's disease, is caused by *M paratuberculosis*. Dalziel first drew attention to the histological similarity between Johne's disease in cattle and what was later to be called Crohn's disease in humans.² Since the first report of this isolation of *M paratuberculosis* from humans in 1984,⁷ it has been isolated from seven patients in three different centres. The importance of these results and of serological studies, however, remains unclear.⁸ Thayer *et al* found that the antibody response to *M paratuberculosis* in patients with Crohn's disease was significantly higher than in normal controls.⁹ In another study, however, no consistent IgG, IgM, or IgA antibody response was found in Crohn's disease patients.¹⁰ Because the antigen preparation that was used in the two studies was different, comparison is difficult. In a study of monkeys

infected with *M paratuberculosis*, those without clinical disease had antibodies to *M paratuberculosis*, whereas those with disease did not.¹¹ In an attempt to resolve this controversy we developed an ELISA to detect serum IgG and IgM concentrations to protoplasmic and surface antigen of *M paratuberculosis* in patients with inflammatory bowel disease. In addition we have used an immunoblotting technique to identify mycobacterial antigens to which serum antibodies are directed and an immunocytochemical method to investigate the possible relation between *M paratuberculosis* and Crohn's disease.

Methods

SUBJECTS

Sera were obtained from 52 patients with Crohn's disease (13 adults, mean age 32 years, range 17-55 years and 39 children, mean age 13.2 years, range 8-16 years); 15 ulcerative colitis patients (eight adults, mean age 38 years, range 20-58 years and seven children, mean age 12.3 years, range 6-16 years); and 41 control patients (23 adults, mean age 37.3 years, range 17-5 years and 18 children, mean age 10.8 years, range 6-16 years).

ELISA FOR M PARATUBERCULOSIS ANTIBODY

M paratuberculosis protoplasmic antigen and surface antigen (Allied Laboratories, Ames, Iowa, USA) were used as antigens. Sera from infected cows with known high titres of antibody to *M paratuberculosis* were purchased from Allied laboratories and used as positive controls.

ANTISERA TO M PARATUBERCULOSIS ANTIGENS WERE PREPARED IN RABBITS

Protoplasmic antigen or surface antigen (2 mg/ml in distilled water) was dispersed into an equal volume of Freund's incomplete adjuvant. Some 0.5 ml of either antigen preparation was injected intramuscularly into the hind leg of a female New Zealand white rabbit of about 2.5 kg. Immunisations were repeated and the rabbits were bled one month and three months after the first injection. Alkaline phosphatase conjugated goat anti-human IgG, IgM (Sigma, Dorset, UK) and rabbit anti-bovine IgG prepared as described by Voller *et al*¹² were used in our assay.

Initial calibration experiments were performed with positive bovine sera, hyperimmune sera, and 25 randomly selected serum samples. Each serum was double diluted over the range 1:2 to 1:1024 and 100 µl volumes were used in the ELISA as described above.

Microtest III plates (Falcon, Becton Dickinson

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son) were coated with mycobacterial antigen at 0.04 mg/ml in carbonate/bicarbonate buffer pH 9.6. The plates were incubated overnight at 4°C and then washed three times with phosphate buffered saline (PBS) pH 7.4 containing 0.05% (v/v) Tween 20 (PBS/T20). Bovine serum albumin (1% w/v) (Sigma, Dorset, UK) in PBS pH 7.4 (PBS/BSA) was added to the wells to reduce non-specific binding. The plates were incubated for two hours at room temperature, washed three times with PBS/T20, and stored at -20°C until needed. One hundred µl of sera diluted 1:50 with PBS/BSA was added to the wells. After incubation overnight at 4°C, the plates were washed three times with PBS/T20. Altogether 100 µl of conjugate diluted 1:1000 for anti-human Ig and 1:400 for anti-bovine Ig respectively with PBS/BSA was added to the wells. The plates were incubated for two hours at room temperature and washed three times with PBS/T20. A total of 100 µl 1 mg/ml alkaline phosphatase substrate (Sigma 104) in substrate buffer pH 8.9 consisting of 0.2 M NaCO₃, 0.2 M NaHCO₃, 0.01M MgCl₂ was added and incubated for one hour at room temperature. The reaction was stopped with the addition of 100 µl of 1N NaOH and the colour was read spectrophotometrically at 405 nm using a BioRad Model 2550 EIA reader (BioRad).

IMMUNOBLOTTING

Antigen preparations were dissolved in sodium dodecyl sulphate and electrophoresed in 10% polyacrylamide gels in a discontinuous buffer system at a constant current of 15 mA. Proteins were transferred onto nitrocellulose paper and blotted by previously described methods.

IMMUNOCYTOCHEMISTRY

Immunostaining for *M paratuberculosis* was performed by the indirect peroxidase method, using peroxidase conjugated swine anti-rabbit immunoglobulins (Dako Ltd, UK) and diaminobenzidine as chromogen. Endogenous peroxidase was inhibited by treatment with methanol/hydrogen peroxide and non-specific binding of the primary antiserum was blocked by dilution in normal swine serum. Initially, sections were prepared from formalin fixed *M paratuberculosis* which had been pelleted into low melting temperature agar and processed to paraffin wax. Subsequently, wax sections of tissue from patients with histologically diagnosed Crohn's disease (n=18), ulcerative colitis (n=10), and non-inflammatory bowel disease control subjects (n=10) were similarly stained. Positive control sections (wax embedded *M paratuberculosis*) and

negative control sections (non-immune rabbit serum) were also performed. Sections were examined at high power magnification (×40 objective).

Results

SERUM ANTIBODY RESPONSE

The mean and range of optical density values obtained are shown in the Table. In each patient group there was a wide variation in the concentrations of antibody detected. Patients with Crohn's disease did not have significantly higher IgG or IgM antibody concentrations to either antigen preparation than the non-inflammatory bowel disease control patients or patients with ulcerative colitis. There was no correlation between the concentration of *M paratuberculosis* antibody and the duration of disease illness in the Crohn's disease patients (results not shown).

IMMUNOBLOTTING

Seven major bands were detected whose apparent molecular weights varied from 17 to 50 kD. No specific band or band pattern was produced with either antigen, which distinguished the Crohn's disease patients from those with ulcerative colitis, or the non-inflammatory bowel disease patients (results not shown). There was a strong IgG response against a 45 kD antigen from the *M paratuberculosis* protoplasmic antigen preparation in four of 10 Crohn's disease patients, and it was also weakly present in two other patients. However, a weak response to the same antigen was also given by four ulcerative colitis and one of the non-inflammatory bowel disease patients. It is therefore unlikely to be of diagnostic use.

DETECTION OF M PARATUBERCULOSIS IN TISSUE SPECIMENS USING IMMUNOCYTOCHEMISTRY

Immunocytochemical analysis of wax sections of formalin fixed *M paratuberculosis* showed positive staining with each antiserum. No staining was observed when normal rabbit serum replaced the primary antisera, indicating the specificity of the method. The presence of *M paratuberculosis* was not detected in any patient with Crohn's disease or ulcerative colitis.

Discussion

The serology of mycobacterial infections was recently described as a quagmire.⁸ Thayer *et al* reported increased concentrations of antibodies to *M paratuberculosis* protoplasmic antigen in patients with Crohn's disease, compared with patients with ulcerative colitis or a healthy control group. In another study, however, Sang-Nae Cho *et al*,¹⁰ using a different antigen preparation, failed to find a consistent IgG, IgM, or IgA antibody response in patients with Crohn's disease. In our study, we have used the same protoplasmic antigen preparation as Thayer *et al*⁹ together with a second surface antigen preparation. Although we were able to show IgG and IgM antibody activity in patients

IgG and IgM antibody response to *M paratuberculosis* antigens in different patient populations measured by an ELISA

Patient group	Mean (SD) optical density (460 nm)			
	Surface antigen		Protoplasmic antigen	
	IgG	IgM	IgG	IgM
Crohn's disease (n=52)	0.67 (0.05)	0.40 (0.14)	0.50 (0.38)	0.19 (0.11)
Ulcerative colitis (n=15)	0.26 (0.20)	0.45 (0.46)	0.34 (0.18)	0.20 (0.17)
Non-inflammatory bowel disease (n=41)	0.47 (0.24)	0.36 (0.22)	0.59 (0.32)	0.25 (0.13)

with Crohn's disease, antibody concentrations were similar in patients with ulcerative colitis and a control group without inflammatory bowel disease.

The antigen preparations used in the present study almost certainly contained a variety of different antigens, and therefore the ELISA measured the total antibody response to these antigens. It is possible that the antibody response to one of the antigenic components may be specific to Crohn's disease.

Immunoblotting, on the other hand, provides information on each antigenic component separately. No band(s) was detected which was specific to or more intense in patients with Crohn's disease. Although the 47 kD antigen seemed dominant in four of 10 Crohn's disease patients, this antigen was also found in patients with ulcerative colitis indicating that it is of no diagnostic value.

Direct detection of *M paratuberculosis* using an in situ immunoperoxidase technique has been reported in experimental infections of goats,¹³ but this has not been confirmed in studies of Crohn's disease. We failed to detect any *M paratuberculosis* antigens in intestinal tissue sections from 18 patients with Crohn's disease.

The results of this study do not support an

aetiological role for *M paratuberculosis* in Crohn's disease.

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