

Mycobacterial aetiology of Crohn's disease: serologic study using common mycobacterial antigens and a species-specific glycolipid antigen from *Mycobacterium paratuberculosis*

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SUMMARY Crohn's disease is a granulomatous form of enteritis superficially similar to Johne's disease (paratuberculosis) of ruminants. Recently, a *Mycobacterium* sp closely related to *Mycobacterium paratuberculosis* was cultured from tissues of patients with Crohn's disease suggesting that *M paratuberculosis* may be the aetiologic agent in some cases. In addition, greater seroreactivity to *M paratuberculosis* has been reported in patients with Crohn's disease. In the present study, we have evaluated the serum antibody response to disrupted *M paratuberculosis* using ELISA and serum specimens from 33 people with Crohn's disease, 21 with ulcerative colitis, and 12 non-inflammatory bowel disease controls. We failed to find a consistent IgG, IgM, or IgA antibody response to *Mycobacterium paratuberculosis*. The results indicate that, as in bovine paratuberculosis, serum seroreactivity is not a reliable tool for examining the relationship between human intestinal diseases and mycobacteria.

Chiodini *et al* recently reported the isolation from the tissue of patients with Crohn's disease of a *Mycobacterium* sp that proved to be a strain of *Mycobacterium paratuberculosis* both by biochemical¹ and genetic² parameters. They also reported a significant increase in antibody titre to *M paratuberculosis* antigens in sera from Crohn's disease patients as compared with controls or patients with ulcerative colitis.³

The relationship between Crohn's disease and mycobacteria is long, and one which is periodically resurrected. For example, Blaser *et al*⁴ studied the seroreactivity against arabinomannan in Crohn's disease patients and found no appreciable difference as compared with controls. Nor was an association observed between seroreactivity and the Crohn's disease activity index or the duration of illness. Grange *et al*⁵ had reported increased levels of total

immunoglobulin A in British patients with Crohn's disease similar to those seen in British patients with sarcoid or in Indonesian controls. Interestingly, Grange *et al* did note that British Crohn's disease patients had high antibody levels against an ultra-sonicate of BCG. The possibility remains, however, that the raised total IgA levels portend an increased antigenic stimulus from intestinal antigens, a possibility which is consistent with the report of Blaser's *et al*⁴ that Crohn's disease patients had raised responses to common intestinal bacteria.

We have attempted to duplicate the results of Thayer *et al*³ and in addition test for the presence of antibodies to a specific glycolipid antigen present in some strains of *M paratuberculosis*.⁶

Methods

ANTIGEN

Mycobacterium paratuberculosis, National Animal Disease Center strain 18, was grown on 7H9 medium⁷ at 37°C, harvested by centrifugation, heat

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killed and partially disrupted by probe sonication. This total sonicate was used as whole cell antigen. The *M paratuberculosis* strain 18 specific glycolipid antigen, polar glycopeptidolipid (GPL)-I, with a characteristic 2,3-di-o-methyl- α -L-fucopyranosyl (1-3) rhamnopyranosyl non-reducing terminus, was purified to thin layer chromatographic homogeneity⁶ and used as a species-specific solid phase antigen.

SERUM SPECIMENS

Sera were obtained from 33 patients with Crohn's disease, 21 with ulcerative colitis, and 12 non-inflammatory bowel disease controls (10 duodenal ulcer patients, one with diverticulitis, and one with colon cancer). The majority of the inflammatory bowel disease patients were those seeing Dr Korelitz and these patients had clinically active disease.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The indirect ELISA protocol of Voller⁸ was used with the modifications described previously.⁹ Whole cell sonicate (20 μ g/ml) or polar GPL-I (2 μ g/ml) was suspended in carbonate-bicarbonate buffer, pH 9.6, by sonication, and 50 μ l added to U-bottom wells of polystyrene microtitre plate (Dynateck, Alexandria, VA), and incubated at 37°C overnight in a moist chamber. After washing the wells with phosphate buffered saline (PBS) pH 7.4, containing 0.1% (v/v) Tween 80 (PBST), 100 μ l PBST containing 1% (w/v) bovine serum albumin was added as a second blocking reagent, and the plate was incubated at 37°C for one hour. After decanting the blocking solution, 50 μ l serum specimens diluted 1:100 in PBST, containing 20% (v/v) normal goat serum (NGS), were added to individual wells and incubated at 37°C for one hour. After washing wells with PBST, 50 μ l of peroxidase-conjugated anti-human IgG, IgM, or IgA (Cappel Laboratories, Downingtown, PA) diluted (1:1000) in PBST-20% NGS was added and the plate incubated at 37°C for

one hour. After washing with PBST, 50 μ l substrate solution (1-phenylenediamine) was added, and plates incubated at room temperature for 15 minutes in a dark chamber. Reaction was stopped by the addition of 50 μ l 2.5 N H₂SO₄, and absorbances were read at 490 nm. The absorbance value for each test serum was adjusted by first subtracting antigen and antibody control values before evaluation. The mean A₄₉₀ and standard deviation of the antigen control, which consisted of antigen, diluent, peroxidase, conjugated antihuman immunoglobulin, and substrate, was 0.101 \pm 0.017 for antihuman IgG, 0.180 \pm 0.022 for antihuman IgM, and 0.092 \pm 0.014 for antihuman IgA, respectively. A serum was considered positive if the optical density was greater than the mean plus three standard deviations of that obtained with a panel of 34 sera of known low absorbance obtained from personnel at Colorado State University and routinely used as negative controls in other analogous assays.⁹

Results

Results of analysis of seroreactivity against the common mycobacterial antigens, as presented in the form of disrupted bacilli, are summarised in the Table. Activity was evaluated for each of the immunoglobulin isotypes in serum specimens from each population group. Overall, there was no significant difference between controls, patients with ulcerative colitis, or those with Crohn's disease. No correlations were observed between the per cent of Crohn's disease patients with positive reactions to whole cell antigen, the site - (that is, whether the small bowel, colon or both were involved) duration, clinical severity, or treatment. Finally, there was no observable difference in seroreactivities among the various groups when the pure glycolipid antigen was included in the assay. In fact, unlike when the whole cell antigen was used, none of the serum specimens tested, whether control or from patient, displayed

Table 1 Results of ELISA* for antimycobacterial antibodies in Crohn's disease patients and controls

Group	Patients (n)	Antibody isotype					
		IgG		IgM		IgA	
		% positive	A ₄₉₀ †	% positive	A ₄₉₀	% positive	A ₄₉₀
Controls	12	50	0.639	25	0.163	58	0.150
Ulcerative colitis	21	43	0.556	29	0.252	19	0.094
Crohn's	33	36	0.618	33	0.229	18	0.112

*Details of ELISA are described in the text. Each serum was diluted 1:100. A serum was considered positive if the optical density (A₄₉₀) was >mean+3 SD of the 34 serum samples with low absorbance.

†Mean of absorbance (490 nm) for group in ELISA.

demonstrable activity against the *M paratuberculosis* glycolipid. Accordingly, no data are shown for the glycolipid.

Discussion

Mycobacteria may be environmental organisms so that human exposure is common, and serum antibodies to mycobacteria are ubiquitous in blood from mammalian and avian species.^{3 10 11} In addition, mycobacteria contain a large variety of powerful immunogens that are shared between both environmental and pathogenic organisms^{11 12} and with other bacterial species – for example, arabinomannan is a common mycobacterial antigen present in all species of mycobacteria as well as in nocardia and corynebacteria.^{12 13} Other antigens are specific or, indeed, sub-species-specific,^{13 14} and notable among these are a variety of glycolipids, the most recent of which is the 2,3-di-o-methyl-L-rhamnopyranose-containing 'C-mycoside'-glycopeptidolipid from *M paratuberculosis*.⁸ The emergence of this glycolipid, combined with the implication of *M paratuberculosis* in Crohn's disease, promised the type of specific serodiagnosis recently accomplished in the context of leprosy.¹⁴

A previous study³ reported increased seroreactivity to mycobacterial antigens among Crohn's disease patients. We used the same antigen but could not confirm that observation. The differences between the two studies deserve comment. Unfortunately, the previous study did not provide sufficiently detailed methods for direct detailed comparisons – for example, whether diluted or undiluted sera were used. More importantly, the criteria used for scoring a serum sample as positive were not provided. In general, ELISA tests are scored as positive or negative based on the absorbance of known negative controls evaluated simultaneously. A positive test is defined as an optical density significantly greater than that of the control sera (usually greater than the mean plus 3 standard deviations) or as a P/N ratio (positive:negative) greater than 2. We used the method of the OD+3 SD after correcting readings for the non-specific reactions commonly present. In this context, it is important to note that most commercial antisera contain antimycobacterial antibodies as Freund's adjuvant is used in the preparation. Accordingly, use of such reagents results in falsely high absorbance values which must be subtracted before analysis. In addition, in the previous work, the reported raised antibody titres were based on the assumption that they were directly proportional to the increase in optical density achieved. Antibody titre is generally determined by titration of sera, and the titre is

scored as the highest dilution giving a positive test. Differences are usually expressed as differences in geometric mean titre. Use of OD value to establish antibody titre is usually unwise, especially when the reaction is a polyclonal antibody response to a complex group of antigens. Experimentally determined OD values do not reflect titre because the OD value is greatly influenced by the affinities of the individual antibody present, and these may vary greatly.¹⁵

Finally, the earlier work based statistical analyses on difference obtained by applying *t* tests to the optical density values. The use of the *t* test to assess statistical significance was not justified in the circumstances. Indeed, from the data presented in this earlier work, it is impossible to determine whether there was actually any difference between controls and patients with Crohn's disease.

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References

- 1 Chiodini RJ, Van Kruiningen HJ, Thayer WR, Merkal RS, Coutu JA. Possible role of mycobacteria in inflammatory bowel disease. An unclassified Mycobacteria species isolated from patients with Crohn's disease. *Dig Dis Sci* 1984; **29**: 1073–9.
- 2 Yoshimura HH, Merkal RS, Graham DY. DNA hybridization studies of the relationship of *Mycobacterium paratuberculosis* to other mycobacteria and to an isolate from a Crohn's disease patient. [Abstracts of the 85th annual meeting]. Washington, DC: American Society for Microbiology, 1985: 103.
- 3 Thayer WR, Coutu JA, Chiodini RJ, Van Kruiningen HJ, Merkal RS. Possible role of mycobacteria in inflammatory bowel disease. Mycobacterial antibodies in Crohn's disease. *Dig Dis Sci* 1984; **29**: 1080–5.
- 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.
- 5 Grange JM, Gibson J, Nassau E. 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.
- 6 Camphausen RT, Jones RL, Brennan PJ. A glycolipid antigen specific to *Mycobacterium paratuberculosis*: structure and antigenicity. *Proc Natl Acad Sci USA* 1985; **82**: 3068–72.

- 7 Cohn ML, Waggoner RF, McClatchy JK. The 7H11 media for the cultivation of mycobacteria. *Am Rev Respir Dis* 1968; **98**: 295-6.
- 8 Voller A, Bidwell DE, Bartlett A. Procedures. In: *The enzyme linked immunosorbent assay (ELISA)*. Alexandria, VA: Dynatech Laboratories, 1979: 23-125.
- 9 Cho S-N, Yanagihara DL, Hunter SW, Gelber RH, Brennan PJ. Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. *Infect Immun* 1983; **41**: 1077-83.
- 10 Abou-Zeid C, Harboe M, Sundsten B, Cocito C. Cross-reactivity of antigens from the cytoplasm and cell walls of some corynebacteria and mycobacteria. *J Inf Dis* 1985; **151**: 170-8.
- 11 Ratledge C, Stanford J, eds. *The biology of the mycobacteria*. Vol 2. *Immunological and environmental aspects*. New York: Academic Press, 1983.
- 12 Goren MB, Brennan PJ. Mycobacterial lipids: chemistry and biologic activities. In: Youmans GP, ed. *Tuberculosis*, Philadelphia: Saunders, 1979: 63-193.
- 13 Brennan PJ. New-found glycolipid antigens of mycobacteria. In: Lieve L, Schlessinger D, eds. *Microbiology 1984*. Washington, DC: Am Soc Microbiol, 1984: 366-75.
- 14 Hunter SW, Fujiwara T, Brennan PJ. Structure and antigenicity of the major glycolipid antigens of *Mycobacterium leprae*. *J Biol Chem* 1982; **257**: 15072-8.
- 15 Lehtonen O-P, Eerola E. The effect of different antibody affinities on ELISA absorbance and titer. *J Immun Meth* 1982; **54**: 233-40.