SHORT ARTICLE

No evidence for antibodies to mycobacterial A60 antigen in Crohn's disease sera by enzyme-linked immunoabsorbent assay (ELISA)

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Summary. Sera from patients with Crohn's disease were tested with an ELISA system for antibodies to antigen A60 prepared from *Mycobacterium bovis* (BCG), *M. avium* and *M. paratuberculosis*. No evidence was found for significantly high titres of antibody to these antigens.

Introduction

Mycobacteria have been implicated as possible aetiological agents in Crohn's disease (Burnham *et al.*, 1978). Recently, a slow-growing mycobactin-dependent mycobacterium was isolated from Crohn's disease tissue (Chiodini *et al.*, 1984). The organism was similar in its cultural and biochemical properties to *Mycobacterium paratuberculosis*—the causative agent of Johne's disease in ruminants—and has been shown by DNA hybridisation (McFadden *et al.*, 1987a) and by use of DNA probes that distinguish between *M. paratuberculosis* and *M. avium*, to be indistinguishable from *M. paratuberculosis* (McFadden *et al.*, 1987b). Thayer *et al.* (1984) used an enzyme-linked immunoabsorbent assay (ELISA) to detect antibody to a crude preparation of *M. paratuberculosis* antigen and found increased antibody titres to the antigen in 23% of Crohn's disease patients. Also, Grange *et al.* (1980), also using an ELISA test, had found increased antibody titres to a soluble crude antigen preparation of *M. tuberculosis* in Crohn's disease sera. In both these studies significant mycobacterial antibody titres were found in some control sera. More recently, Cho *et al.* (1986) found no evidence for increased antibody titres in Crohn's disease sera to either a crude sonicate of, or a purified species-specific glycolipid from, *M. paratuberculosis*. However, in this study, a high proportion (25–58%) of control patients gave positive reactions to the crude sonicate antigen, whereas no sera gave reactions to

the purified glycolipid antigen. Furthermore, no positive disease controls were used, so the significance of the negative results is uncertain.

The use of a purified and characterised antigen preparation could possibly provide a more sensitive and discriminating test than those in which crude antigen has been used. Such an assay would be useful as a non-invasive diagnostic test for Crohn's disease. To explore this possibility we have used an ELISA to measure antibody levels to A60 antigen—a membrane glycoprotein that is found in most mycobacteria (Cocito, 1985)—prepared from *M. bovis* (BCG), *M. avium*, and *M. paratuberculosis* in sera from Crohn's disease patients. A similar test with A60 antigen prepared from *M. bovis* (BCG) gave positive titres in a high proportion of sera from tuberculosis patients (C. Cocito, personal communication).

Materials and methods

Sera

Control sera (20) were obtained from patients attending hospital and whose blood had been sent for analysis at the National Center for Blood Transfusion (Strasbourg). For this group, sera from patients attending the Pneumology Department were discarded in order not to introduce potential positives. Tuberculosis sera (12) were obtained from patients attending a pneumology clinic (Paris). Three reference tuberculosis sera were samples previously characterised as giving strongly positive, positive and weakly positive optical densities in ELISA with *M. bovis* (BCG) A60 antigen. A negative reference

serum was previously characterised as giving no reaction against the A60 antigens tested. Crohn's disease sera (20) were obtained from patients being treated at the Department of Surgery, St George's Hospital Medical School, London. Most patients had clinically active disease.

ELISA assays

A60 antigens were prepared as described by Cocito (1985) from *M. bovis* strain BCG, *M. paratuberculosis* strain 2E, and *M. avium* strain D4. The antigens were bound to microtitration plates at pH 9.0 in carbonate buffer (Cocito, 1985). Reference sera were introduced into each experiment—one negative serum and three positive sera in order to standardise the tests for day to day fluctuations. The unknown and reference sera were added (100 µl) at a dilution of 1 in 100 and incubated for 60 min at room temperature. The plates were washed and bound antisera were detected with affinity purified chicken anti-human IgG conjugated to peroxidase (100 µl, 60 min at room temperature). Substrate (ortho-phenylene diamine) at pH 5.5 plus H₂O₂ was added, incubated for 15 min at room temperature and the optical density values were read at 492 nm by a Titertek Multiscan at 492 nm. The readings were scored as negative (–), weakly positive (+/–), positive (+), or strongly positive (++) by comparison with the reference sera.

Results

Sera from the patients with Crohn's disease were first tested for antibody against A60 antigen prepared from *M. bovis* (BCG), and *M. paratuberculosis* (table I). Only one of the Crohn's disease sera (no. 6) yielded a significant OD against the antigens tested, showing identical reactivity against A60 antigen from *M. bovis* (BCG) and *M. paratuberculosis*. Three other antisera (nos. 2, 4 and 20) gave weaker, but not significant values. The patients whose sera yielded positive values could not be distinguished from the others on the basis of disease activity, site of involvement, duration, or treatment of disease. Sera from the control group gave a similar pattern of reactivity against the *M. bovis* (BCG) A60 antigen, although three had significantly positive results (table II).

The same Crohn's disease sera were next tested for antibody against A60 antigen purified from *M. avium* and twelve tuberculosis sera were tested in the same experiment. The results are shown in table III. Tuberculosis sera, as expected, gave positive reactions with the *M. bovis* (BCG) A60 antigen, but also gave positive results with *M. avium* A60 antigen. The Crohn's disease sera presented a similar pattern of reactivity with *M. avium* A60

Table I. Reactivity of Crohn's disease sera with A60 antigen prepared from *M. bovis* (BCG) and *M. paratuberculosis* in an ELISA

Serum no.	Reaction of serum with A60 antigen from			
	<i>M. bovis</i>		<i>M. paratuberculosis</i>	
	OD	Classification	OD	Classification
Reference sera				
Neg.	0.07	–	0.08	–
TB1	0.205	+ / –	–	–
Crohn's sera				
1	0.03		0.04	
2	0.07		0.24	+ / –
3	0.00		0.03	
4	0.24	+ / –	0.17	
5	0.05		0.08	
6	0.37	+	0.37	+
7	0.07		0.03	
8	0.02		0.04	
9	0.01		0.00	
10	0.03		0.08	
11	0.00		0.00	
12	0.00		0.00	
13	0.01		0.02	
14	0.02		0.08	
15	0.00		0.08	
16	0.06		0.11	
17	0.12		0.18	
18	0.11		0.16	
19	0.02		0.04	
20	0.16		0.21	+ / –

antigen as with *M. bovis* (BCG) and *M. paratuberculosis* A60 antigens. Sera nos. 4 and 6 were positive and nos. 2, 3, 10 and 20 showed weak reactivity.

Discussion

No evidence was found for significant levels of reactivity of Crohn's disease sera with A60 antigen purified from *M. avium*, *M. bovis* or *M. paratuberculosis*. The reference tuberculosis sera gave positive values in each test and the control sera gave few positives, demonstrating the specificity of the test. The positive values obtained for the Crohn's and control sera could have been due to previous exposure to *M. tuberculosis* or other environmental mycobacteria. Similar tests with sera from tuberculosis patients gave positive values against A60 antigen prepared from *M. tuberculosis* with most sera tested (M. Baelden, personal communication). In addition, the results with the tuberculosis sera included in table III indicate that the test is positive even when the test antigen (*M. avium*) differs from the sensitising antigen (*M. tuberculosis* or *M. bovis*),

Table II. Reactivity of control sera with A60 antigen prepared from *M. bovis* (BCG)

Serum no.	Reactivity with <i>M. bovis</i> A60	
	OD	Classification
Reference sera		
Neg.	0.25	—
TB1	0.35	+/-
TB2	0.69	+
Control sera		
1	0.21	
2	0.25	
3	0.49	+
4	0.20	
5	0.22	
6	0.15	
7	0.50	+
8	0.37	+/-
9	0.17	
10	0.15	
11	0.17	
12	0.48	+
13	0.20	
14	0.29	
15	0.18	
16	0.29	
17	0.45	+/-
18	0.36	+/-
19	0.18	
20	0.15	

showing a high degree of cross reactivity to A60 antigen.

These results demonstrate that Crohn's disease patients do not have increased levels of antibody to A60 antigen prepared from *M. bovis*, *M. paratuberculosis* or *M. avium*. The results also show that in a known mycobacterial disease (tuberculosis), sera cross-react with antigen prepared from *M. avium* and *M. paratuberculosis*. It seems unlikely, therefore, that the lack of reactivity of Crohn's disease sera with the A60 antigens could be due to insufficient cross-reactivity of Crohn's disease-involved mycobacteria with the tested antigens. This does not however exclude the possibility of mycobacterial involvement in Crohn's disease. Many patients may be anergic to the mycobacterial antigens as sometimes occurs in leprosy. Alternatively, A60 antigens may not be exposed to the immune system in Crohn's disease, so that patients may not develop antibody. When mycobacteria were isolated from Crohn's-disease tissue they first appeared as extremely slow-growing, possibly cell-wall defective forms (Burnham *et al.*, 1978; Chiod-

Table III. Reactivity of Crohn's disease and tuberculosis sera with A60 antigen prepared from *M. avium*

Serum no.	Reactivity with <i>M. avium</i> A60	
	OD	Classification
Tuberculosis sera		
1	1.58	++
2	0.61	++
3	0.41	+
4	0.88	++
5	0.72	++
6	0.66	++
7	0.96	++
8	1.00	++
9	1.05	++
10	1.31	++
11	1.20	++
12	1.26	++
Reference sera		
Neg.	0.15	—
TB2	0.42	+
TB3	1.35	++
Crohn's sera		
1	0.22	—
2	0.33	+/-
3	0.30	+/-
4	0.65	+
5	0.27	—
6	0.54	+
7	0.21	—
8	0.21	—
9	0.23	—
10	0.30	+/-
11	0.27	—
12	0.21	—
13	0.17	—
14	0.20	—
15	0.18	—
16	0.27	—
17	0.21	—
18	0.22	—
19	0.21	—
20	0.39	+/-

ini *et al.*, 1984). These forms may not express A60 antigen. The A60 antigen of *M. bovis* is preponderant during exponential multiplication and recedes during declining phases (Cocito, 1985). Also, sera from tuberculosis patients in a progressive phase of the disease react readily in this system (C. Cocito, personal communication).

The results presented here suggest that in the experiments described by Thayer *et al.* (1984) and Grange *et al.* (1980), who obtained significant antibody levels in Crohn's disease sera with *M.*

paratuberculosis and *M. tuberculosis* crude cell homogenates as antigen sources, the reacting antigen is unlikely to have been the A60 glycoprotein; therefore, an ELISA test based on the A60

glycoprotein has no value in the diagnosis of Crohn's disease.

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