

SEROREACTIVITY OF CROHN'S DISEASE PATIENTS TO MYCOBACTERIAL ANTIGENS: ORIGINAL DATA AND ANALYTICAL REVIEW OF EARLY LITERATURE

Seroreactividad de pacientes de la enfermedad de Crohn a antígenos micobacteriano: datos originales y revisión de los estudios pioneros

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ABSTRACT

The mycobacterial etiology of Crohn's disease has longtime been the subject of discussion and several studies on this issue have been published. In this paper we set out to critically analyze reports on the humoral response against mycobacterial antigens among Crohn's disease patients and matched samples of ulcerative colitis, tuberculosis, and healthy controls, published during the decade that followed initial suspicions in the early-mid 1980s. We also provide our own previously unpublished results of the humoral response of Crohn's disease, ulcerative colitis, and tuberculosis patients and healthy controls to an adapted paratuberculosis (PPA-3 antigen) ELISA. Our study indicate that statistical analysis of some studies were not adequate, and that a general significantly ($p < 0.01$) increased reactivity against mycobacterial antigens could be observed in the combined analysis of all available data (meta-analysis). The reactivity of Crohn's patients did not significantly differ from reactivity of tuberculosis patients against mycobacterial antigens.

RESUMEN

La etiología micobacteriana de la enfermedad de Crohn es objeto de discusión desde hace mucho tiempo y se han publicado diversos trabajos al respecto. En este artículo se lleva a cabo un análisis crítico de la información generada sobre la respuesta humoral frente a antígenos de *Mycobacterium* en pacientes con enfermedad de

Crohn, colitis ulcerosa y tuberculosis y en controles sanos, durante la década siguiente a las primeras sospechas a comienzos de 1980s. Además incluimos resultados de nuestro laboratorio no publicados anteriormente, sobre la respuesta humoral de pacientes con enfermedad de Crohn, colitis ulcerosa y tuberculosis y en controles sanos a un ELISA con el antígeno PPA-3 de paratuberculosis. Nuestro estudio de meta-análisis indica que el análisis estadístico de los datos en alguno de los estudios realizados no fue adecuado y que globalmente los pacientes de Crohn tienen una respuesta significativa a antígenos de micobacterianos, que es similar a la de pacientes de tuberculosis ($p < 0,01$).

INTRODUCTION

Crohn's disease is a granulomatous ileocolitis of unknown etiology. Current concepts on the immunopathogenesis of Crohn's disease suggest that disease results from an initial or persistent "insult" to the gut-associated lymphoid tissues (GALT), which through the liberation of cytokines and other inflammatory mediators, sets the stage for a chronic and persistent abnormal inflammatory response.

The leading theory for this abnormal inflammatory reaction within the intestine is that Crohn's disease is the result of immunologic hyper-responsiveness of the intestinal immune system to constituents arising from within the intestine. This hyper-reactivity might result from an immune defect or a persistent stimulus. The possibility exists that disease results from a non-specific outcome of disordered mucosal immune regulation, with uncontrolled hyper-reactivity to exogenous antigens based on a defective down-regulation of the response. Genetic predisposition and exogenous triggers might operate at the level of the "target" or the mucosal immune response.

Since the early to mid-1980's, it has been suggested that the immunologic trigger or persistent stimulus was caused by *Mycobacterium paratuberculosis*, the etiologic agent of paratuberculosis or Johne's disease in ruminant animals. This suggestion has arisen from the isolation of *M. paratuberculosis* from 2 to 15% of Crohn's disease patients⁴, the detection of *M. paratuberculosis* DNA (IS900) in up to 65% of patients with Crohn's disease¹⁴, demonstration of

the ability of these *M. paratuberculosis* isolates to cause granulomatous intestinal disease in experimental animals, and the similarities to other known mycobacterioses. Despite a great deal of effort, this hypothesis has neither been proved nor disproved and remains a controversial subject.

One of the quandaries of the hypothesis relates to the failure to consistently demonstrate an immune response to *M. paratuberculosis* in Crohn's disease patients. Crohn's disease is generally accepted to be an immunologic abnormality in response to a stimulus, and if *M. paratuberculosis* is etiologically related, a response must be demonstrable. Several studies have addressed the issue by investigating the presence of antibodies against paratuberculosis in Crohn's disease patients. About half of them found no differences in the level of antibodies between Crohn's disease patients and controls, while in the other half, significant differences have been reported. Although a variety of possibilities exist for these discrepancies and failure to consistently demonstrate a response, the enigma created by these conflicting findings persists.

These conflicting reports led us to carry out a serological study in the Basque Country and to perform meta-analysis and critical review of previous published data in an attempt to clarify these discrepancies. It was found, in original data as well as in previously published data, that a disproportionate number of Crohn's disease patients have a significantly increased humoral response to *M. paratuberculosis* antigens.

MATERIALS AND METHODS

Original data

A total of 197 human sera were obtained from two hospitals in the Basque Country which included 66 Crohn's disease (CD) patients, 52 ulcerative colitis (UC) patients, 31 active pulmonary tuberculosis (TB) patients and 48 healthy blood donor controls (HC). Patients were assigned into each group based on diagnostic criteria as defined in Table 1. Sera were collected during a three year period and frozen at -20°C until processed.

Sera were analyzed by an enzyme linked immuno-sorbent assay (ELISA) using *M. paratuberculosis* PPA-3 antigen (Allied Monitor, Inc., Fayette, MO). Assays were performed as previously described¹. Briefly, microtiter plates were coated with 100 µl of antigenic solution (0.04 mg/ml⁻¹ PPA-3) per well by overnight incubation at 4°C and stored at -20°C until use. Each serum sample was randomly allocated to one of five batches in order to insure that all groups were equally represented in each plate.

An arbitrarily chosen control serum was also included in duplicate in each plate to control inter-plate variability. One hundred microliter of each serum was adsorbed with a suspension of *Mycobacterium phlei* (1:1) by 2 h incubation at room temperature. Adsorbed sera were diluted 1 to 100 in PBS-tris-glycerine (PBS-TG), and 100 µl were added to each of two wells. Plates were incubated for 2 h at room temperature and then washed with PBS-TG. Anti-human IgG rabbit horseradish peroxidase conjugate (1:4500) was added to each well and incubated for an additional 2 h at room temperature. 2,2'-azino-di-ethyl-benzy-thiazoline sulfonate (ABTS) was added, and the reaction was stopped after incubation for 20 min in the dark at room temperature. Optical density was determined at 405 nm (OD₄₀₅).

Analysis of variance was performed on the OD results to determine the immune response in each group and the significance of the differences between group means was determined by a Dunnett test. Since this type of analysis only shows differences in the mean reactivity irrespective of clinical significance, a

Table 1. Characteristics of the groups of disease in the current study.

		Crohn's Disease	Ulcerative colitis	Pulmonary Tuberculosis
Diagnostic criteria	Biopsy	30	45	0
	Intestinal transit test	14	0	0
	Colonoscopy	4	6	0
	Radiology	5	0	0
	Sputum culture	0	0	31
	Unknown	0	1	0
Activity of the disease	Active	25	21	31
	Inactive	27	31	0
	Unknown	14	0	0
Sex	Female	27	21	12
	Male	26	32	19
Age	Median	34	43	34
	Range	14-87	20-91	19-81

positive threshold was fixed at three standard deviations above the mean, and all the sera were classified as positive or negative accordingly. A one-tailed Fisher exact test was performed to determine differences between the frequencies of positives in each group.

Meta-analysis of other data

Data from published literature which contained enough information to determine the number of observations and the mean and standard deviations of anti-*Mycobacterium paratuberculosis* IgG were pooled weighing the mean proportionally to the quotient of the number of observations in each report to the total number of observations. Significance of the differences between control group and disease groups were estimated according to standard statistical methods¹⁶ using a variance estimate for stratified sampling because this method yielded the more conservative results. Briefly, the difference between each pair was weighed proportionally to the number of observations in each report relative to the total number of observations. Variance was determined proportionally to the square of each group's weight relative to the total number of observations. When data provided by authors corresponded to different phases of disease activity, the groups were pre-pooled using the same weighing method. The probability of the higher value of Student's t defining a range of variation of the difference between means not including zero was taken as the level of significance of such difference.

In addition to this, and to encompass the maximum number of reports, a simplification of the analysis based in reducing quantitative to qualitative data was also used. Thus, any report where number of positive and negative serological results in each group of patients was given or could be calculated was selected for the general analysis. In this case the weighing factor was calculated as the ratio of the reciprocal of

each report variance to the total sum of the reciprocals of all the studies⁸. However, in order to define positive results, different criteria were used according to the manner in which the data were presented in the original papers. Table 1 summarizes the sources of data as well as the manner in which the data were used.

In general, the cut-off for scoring positive results was established at the level at which the highest specificity for discrimination between Crohn's and healthy controls could be obtained. In some cases, corrections on the published data were necessary. For instance, in the paper by Thayer et al.¹⁹, data presented in table form did not agree with figures provided in text, and a typographical error was assumed. Since the figure for the percent positives in the active Crohn's disease group had no decimals, and the 23% of positives given in the text for both active and inactive corresponded to the number of observations above the mean + 2 SD in the graph, it was assumed that 13 was the absolute number of positive reactors in the former group. Therefore, according to the author's cut-off, a total of 16 positives were observed for both active and inactive Crohn's, as compared to 9 positives in both PPD positive and negative controls.

Data from the paper by Brunello et al.¹, was subjected only to qualitative analysis because the data were provided only graphically, and that allowed only a categorical analysis based in moving the cut-off line. However, no numerical data was available for qualitative analysis. Therefore, by setting the cut-off value 0.05 OD units (11%) above the value used by the authors lead to 4 positives in the Crohn's disease group and none in the controls. This small change greatly improved the discriminating value of the test, in contrast to the statistical criteria (mean + 3 x SD) used by the authors.

The work by Kobayashi et al.⁹ also found no association between Crohn's disease and paratuberculosis antigens. These conclusions appeared to be derived from an unusually

Table 2. Summary of the published information on anti-mycobacterial humoral responses of Crohn's patients and its use in this study.

Reference	Method	Antigen	Comparison of means	Comparison of proportions
Matthews et al., 1980 ¹¹	Agglutinat.	Whole cell	No mean and SD	Too many positive controls
Thayer et al., 1984 ¹⁹	ELISA	Protoplasm (Map)	Calculated mean and SD	Modified proportions
Cho et al., 1986 ³	ELISA	Whole cell (St18)	No SD given	Given proportions used
Cho et al., 1986 ³	ELISA	Glycolipid (St18)	No mean and SD given	All groups negative
Kobayashi et al., 1988 ⁹	ELISA	LAM (<i>M. leprae</i>)	No mean and SD given	Modified proportions
Kobayashi et al., 1988 ⁹	ELISA	Protoplasm (Map)	No mean and SD given	Modified proportions
Kobayashi et al., 1988 ⁹	ELISA	Lipid A (<i>S. minnesota</i>)	No Mycobacteria-specific	No Mycobact.-specific
McFadden et al., 1988 ¹²	ELISA	A60 (<i>M. bovis</i>)	Excessive inter-assay var.	Given proportions used
McFadden et al., 1988 ¹²	ELISA	A60 (Maa/Map)	No controls data	No controls data
Tanaka et al., 1991 ¹⁸	ELISA	Protoplasm (Map)	Given mean and SD	No proportions given
Tanaka et al., 1991 ¹⁸	ELISA	Surface antigen (Map)	Given mean and SD	No proportions given
Tanaka et al., 1991 ¹⁸	I-blotting	Not specified (Map)	No quantitative data	Calculated proportions
Markesich et al., 1991 ¹⁰	I-blotting	Stress proteins (Map)	No quantitative data	Calculated proportions
Markesich et al., 1991 ¹⁰	I-blotting	Stress proteins (<i>M. smegmatis</i>)	No quantitative data	Calculated proportions
Brunello et al., 1991 ²	ELISA	Protoplasm (Map)	No mean and SD	Modified proportions
Elsaghier et al., 1992 ⁷	ELISA	Protoplasm (Map), p24	No OD. Only titers	Modified proportions
Elsaghier et al., 1992 ⁷	ELISAc	Protoplasm (Map), p24	No quantitative data	Modified proportions
Elsaghier et al., 1992 ⁷	ELISA	Protoplasm (Map), p18	No OD. Only titers	Modified proportions
Stevens et al., 1992 ¹⁷	ELISA	Mycobacterial rHSP60	No OD. Only indices	Modified proportions
Wayne et al., 1992 ²¹	ELISA	Whole cell (<i>M. tuberculosis</i>)	No mean and SD	Modified proportions
Wayne et al., 1992 ²¹	ELISA	Whole cell (<i>M. avium</i>)	No mean and SD	Modified proportions
Wayne et al., 1992 ²¹	ELISA	Whole cell (<i>M. gordonae</i>)	No mean and SD	Modified proportions
Elsaghier et al. 1992 ⁶	ELISA	Mycobacterial rHSP70	No OD. Only titers	Given proportions
Elsaghier et al., 1992 ⁶	ELISA	Mycobacterial rHSP65	No OD. Only titers	Given proportions
Stainsby et al., 1993 ¹⁵	ELISA	Protoplasm (Map)	Given data used	Given data used
Morgante et al., 1994 ¹³	ELISA	Protoplasm (<i>M. tuberculosis</i>)	Given data used	Given data used
Morgante et al., 1994 ¹³	ELISA	Protoplasma (<i>M. avium</i>)	Given data used	Given data used
Morgante et al., 1994 ¹³	ELISA	Protoplasm (Map)	Given data used	Given data used
El-Zaatari et al., 1995 ⁵	Wblot	Map rHSP65	No mean and SD	Given data used
Walmsley et al., 1996 ²⁰	ELISA	18 kD (Mtb)	No OD. Only titers	Modified proportions

low significance threshold. These authors did not provide sufficient numerical data to independently test their hypothesis; rather, they provided several graphs in which a discriminating line could be traced. We focused on the IgG results for lipoarabinomannan (LAM) and *Mycobacterium paratuberculosis* strain Linda antigens, setting a cut-off at approximately 26 and 13 titer units, respectively, where the best discrimination between Crohn's disease and healthy controls could be achieved. Counting the number of Crohn's disease patients (active and inactive) and controls above this line provided the ability to compare frequencies between the two groups. From this report, only 69 active and inactive Crohn's disease patients were used in order to avoid any assumption on missing values presumed to have been lost in graphic overlap.

A similar approach was used with graphic data provided by Elsaghier et al.⁷. In this case, the cut-off for the p24A antigen was set above the values of all healthy controls which occurred at a titer of ~2.28. Data on the 18-kD antigen was used assuming that the right figure was that given in the text because there was an excess of 10 Crohn's disease sera between the figure and the total number of sera given in text. In the other paper of the group the given cut-offs were used⁶. In the work by Stainsby et al.¹⁵, the cut-off value provided by the authors was used with 8 positives in the Crohn's disease group and 0 in healthy controls. Since these authors carried out several tests using different mycobacterial antigens, only those using pathogenic mycobacteria were selected in order not to put too much weight on a single set of sera.

In the work by Wayne et al.²¹, criteria similar to that used for Kobayashi et al.⁹ was employed. A line was traced in the anti-*M. avium* IgG graph at a point providing a good discrimination between Crohn's disease and controls (~0.2 OD units), and the number of cases above it were counted for Crohn's disease and control groups.

For each set of data, a Fisher exact test was performed to calculate the probability

of obtaining the same or greater number of positives in the Crohn's disease group. In addition, all data were subjected to the same test comparing ulcerative colitis or tuberculosis groups to the control group.

Two sets of data have been analyzed independently. One is the general analysis where all data have been considered no matter what antigen was used. The other is a reduced set where only two datasets are considered in order to homogenize antigens and procedures to include a tuberculosis group.

Finally, upon observing different levels of reactivity between American and European studies, a division of the main study was done in order to test the hypothesis that a different level of reactivity exists between America and Europe regarding mycobacterial antigens.

RESULTS AND DISCUSSION

Original data

ELISA results from both Crohn's disease and the tuberculosis groups had significant differences ($p < 0.01$) as compared to the control group as determined by the Dunnett test (Table 2). The highest reactivity was observed in the tuberculosis group (mean OD=0.230) which only marginally differed ($p < 0.1$) from the Crohn's disease group. The ulcerative colitis group had a mean slightly lower than that of the control group, but which was not significantly different ($p=ns$).

When results were evaluated qualitatively, a significantly higher proportion of positives were observed in the Crohn's disease group than in the controls (Table 3). The tuberculosis group had the highest proportion of positives (32.3%).

Meta-analysis

The first study in which a humoral immune response to *M. paratuberculosis* antigens

Table 3. Quantitative ELISA results of different studies of seroreactivity against mycobacterial antigens.

Group	Statistic	Thayer et al., 1984	Tamaka et al., 1991		Stainsby et al., 1993					Morgante et al., 1994			Current study	Weighed average	
		Map-PPA ¹	Map-PPA	Map-SAP	Min	Mav	Mtb	PPDb	Map-SA ¹	Mtb	Mav	Map-PPA	Map-PPA ²	All antigens and groups	Only Map and TB groups
CD	Mean	0.313**	0.500	0.670	0.610	0.510	0.494	0.075	0.202	0.130	0.070	0.110*	0.210*	0.356**	0.190**
	SD	0.167	0.380	0.050	0.138	0.121	0.130	0.024	0.111	0.120	0.030	0.070	0.067	0.052	0.055
	n	74	52	52	38	38	38	38	53	17	17	17	66	500	83
	CV (%)	53.4	76.0	7.5	22.7	23.7	26.3	32.0	55.0	92.3	42.9	63.6	31.9	14.7	29.1
HC	Mean	0.283	0.590	0.470⁴	0.531	0.466	0.496	0.074	0.200	0.110	0.060	0.060	0.186	0.273	0.120
	SD	0.040	0.320	0.240	0.220	0.206	0.230	0.036	0.065	0.050	0.030	0.070	0.019	0.039	0.038
	n	108	41	41	30	30	30	30	23	52	52	52	48	537	100
	CV (%)	19.2	54.2	51.1	41.4	44.2	46.4	48.7	32.5	45.5	50.0	116.7	10.2	14.4	31.2
UC	Mean	0.240	0.340	0.260	0.503	0.413	0.417	0.090	0.158	0.110	0.060	0.070	0.175	0.215*	0.143
	SD	0.05	0.180	0.200	0.152	0.106	0.118	0.031	0.065	0.070	0.030	0.110	0.035	0.026	0.042
	n	34	15	15	15	15	15	15	10	23	23	23	52	255	75
	CV (%)	20.8	52.9	76.9	30.2	25.7	28.3	34.4	41.1	63.6	50.0	157.1	20.0	12.1	29.1
TB	Mean	--	--	--	--	--	--	--	--	0.590*	0.200*	0.400*	0.230**	0.338**	0.295**
	SD	--	--	--	--	--	--	--	--	0.490	0.160	0.330	0.087	0.136	0.137
	n	--	--	--	--	--	--	--	--	19	19	19	31	88	50
	CV (%)	--	--	--	--	--	--	--	--	83.1	80.0	82.5	37.8	40.1	46.3

CD. Crohn's disease patients; HC. Healthy controls; UC. Ulcerative colitis patients; Map. *M. avium* ssp *paratuberculosis* antigen; PPA. Protoplasmic Antigen; SA. *M. a. paratuberculosis* sonicate; SAP. Surface antigen; NA, Not available; ¹ Both active and inactive disease groups included; ² Sera adsorbed with *M. phlei*; ³ Only data sets obtained with Map antigens and where a matched tuberculosis group is included; ⁴ Not healthy controls, but non-inflammatory bowel disease patients. SD. Standard deviation; n. Number of observations; CV. Coefficient of variation; Comparison to the healthy control group: * p<0.05, ** p<0.01.

was investigated was reported by Thayer et al.¹⁹ These authors concluded that there was a significant difference in reactivity between Crohn's disease and healthy control groups. Shortly after its publication, this report was criticized by Cho et al.³ in which the authors present an argument to discount the data of Thayer et al.¹⁹ The first argument in the Cho et al.³ paper is true: that Thayer et al.¹⁹ failed

to provide some experimental data. However, it can be assumed that experimental conditions were the same for both Crohn's disease and control groups and thus affect both in the same way. The second argument was that Thayer et al.¹⁹ did not provide criterion for scoring positive sera, which appears to overlook the fact that Thayer et al.¹⁹ clearly stated that the positive threshold was set at two times the standard

deviation above the mean of the control group (this detail appears three times in the paper). Regarding the argument of using antisera made with Freund's adjuvant, this could add noise to the ELISA readings but would affect all groups equally. Cho et al.³ group also argues that optical density is not proportional to titer. This is only partially true since there is a lineal relationship between OD readings and the logarithm of the titer. Furthermore, the authors were comparing the values of a continuous variable in two defined populations, not establishing clinical criteria for diagnosis. The statement that 'the use of the *t* test to assess statistical significance was not justified in the circumstances', without any other explanation cannot be sustained, since the Student *t* test is the recommended test for assessing significance of differences between the means of two populations in statistically small samples. Although a more suitable test would have been a comparison of adjusted means after analysis of variance with a *t* test; the direct use of this test with arithmetic means is a common practice in biomedical research. Anyway, it should be born in mind that it is the lack of significant differences what prevents from drawing firm conclusions from statistical analysis because in such a case the statistical risk of doing a wrong decision (no significant difference) is unknown, whereas when the case is the rejection of a hypothesis, the risk is established at a defined level, usually less than 0.05.

The results of Cho et al.³, can be faulted, because either a common mycobacterial antigen or a highly specific one was used. Using a common antigen, Cho et al.³ failed to find any statistical differences. Such conclusion could be explained by the small sample size of the control group and the fact that the antigen was not specific. One or two healthy controls with a reaction due to previous contact with environmental mycobacteria could increase the mean enough as to obscure any difference. This suggestion cannot be tested since the authors

did not present data on variability of results in each group. With the specific antigen, (which turns out not to be specific or even present in *M. paratuberculosis*) no information is provided except that no positive reactions were observed at the fixed threshold of mean + 3 x SD. Any transformation of a continuous variable into a qualitative one results in loss of data; therefore, although at a macroscopic level no reactivity was observed, some degree of reactivity might be found by more thorough analysis. It would be necessary to test the differences between means or search for a threshold that would have provided more information.

Kobayashi et al.⁹ used a rejection level for the hypothesis that Crohn's disease patients have a higher response than controls. Such level actually corresponds to a different hypothesis: that the Crohn's disease patient response is *different than the response of any of the groups*. For testing whether or not antibody levels in Crohn's disease patients are higher than in controls, only a comparison should be made and $p \leq 0.05$ should be the rejection level. A suitable test would have been one in which differences between control group and any other group would have been examined, i.e. four tests, instead of 15. Although no numeric data are provided to properly verify the comparisons, the distribution of the points and the position of the geometric means presented in graphs, suggest that a difference does exist, at least for IgA and IgG with LAM antigen. Simply classifying into positive and negative, demonstrates a significantly higher proportion of reacting individuals in the Crohn's disease group than in controls (Table 3).

The paper by Tanaka et al.¹⁸ is more difficult to explain in relation to our data since it clearly shows that no IgG reactivity could be found in Crohn's disease patients using a PPA antigen. However, the authors omit to explain data presented for surface antigen, for which Crohn's patients appear to show a higher IgG OD mean than controls. In fact, it seems that their PPA

Table 4. Comparison of frequencies of positives obtained in several studies on Crohn's patients seroreactivity against mycobacterial antigens.

Group	America													Europe													TOTAL															
	TPA:12	MPS	GPI	LAMF	MPL:1	Map	Kobayashi et al., 1988	MPL:1	48+65 Map	48+65 Map	Mb	Map ⁶	Wayne et al., 1992	Mb	Map	Morgan et al., 1994	Map	Map	El Zaharti et al., 1995	Total	TPA	Mbo	Map	TPA	MPL ¹	Mbsp65		Mbsp70	Mbsp60	p4 ²	p18	p24 ³	Mm	Mb	Map	PPD ₆	Map	Map	Wainwright et al., 1995	Current Study	Total	
CD	74	33	33	69	69	69	69	34	35	34	22	22	22	22	22	22	16	16	16	13	474	20	10	108	30	28	21	28	28	28	28	38	38	38	38	38	53	33	66	605	1079	444
	% +ve	21.6 ⁴	36.4	0.0	44.9 ⁴	15.9	26.5	57.1	50.0 ⁶	50.0 ⁶	45.5 ⁴	45.5 ⁴	45.5 ⁴	45.5 ⁴	45.5 ⁴	45.5 ⁴	18.8	0.0	12.5	53.9	30.6 ⁶	10.0	60.0 ⁶	3.7 ⁶	50.0 ⁶	14.3	35.7 ⁶	53.6 ⁶	85.7 ⁶	5.3	2.6	5.3	10.5	15.1 ⁴	3.0	13.6 ⁶	18.2 ⁶	23.5 ⁶	18.2 ⁶	23.5 ⁶	18.2 ⁶	
HC	Total	108	12	12	40	40	40	14	12	12	20	20	20	20	20	20	52	52	52	12	466	20	10	149	19	19	17	18	18	18	30	30	30	30	23	20	48	502	968	454		
	% +ve	8.3	50.0	0.0	25.0	17.5	21.4	33.3	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.9	5.8	3.9	41.7	11.4	30.0	100.0	0.0	0.0	10.5	0.0	0.0	0.0	0.0	6.7	3.3	3.3	0.0	0.0	2.1	3.0	7.0	6.4			
UC	Total	34	21	21	28	28	14	15	15	15	23	23	23	23	23	23	23	23	5	235	10	40	20	20	20	20	20	19	20	15	15	15	15	10	12	52	313	548	217			
	% +ve	0.0	42.9	0.0	10.7	10.7	35.7	46.7	46.7	46.7	44.4	0.0	8.7	0.0	12.8	12.8	4.4	0.0	8.7	0.0	12.8	400	2.5	0.0	30.0 ⁶	10.5	0.0	6.7	30.0 ⁶	0.0	6.7	0.0	13.3	0.0	0.0	0.0	7.7 ⁶	9.8	7.8			
TB	Total										19	19	19	4	61	12	34				65.6 ⁶	1000 ⁶																	31	77	138	88
	% +ve										73.7 ⁶	57.0 ⁶	63.1 ⁶	75.0			32.4 ⁶																						41.9 ⁶	46.8 ⁶	53.1 ⁶	44.3 ⁶

CD. Crohn's disease; HC. Healthy controls; UC. Ulcerative colitis PPA. Paratuberculosis protoplasmatic antigen; MPS. *M. paratuberculosis* sonicate; LAM. Liposarbinomannan; MPL. *Mycobacterium paratuberculosis* strain Linda extract; WCA. Whole cell, *M. avium*; p24A. 24-kD protein anodic band; NA. Not available; +/-Total. Number of positives/number of observations; % Percent of positives; ¹ Including active and inactive disease groups; ² Cut-off at mean + 2 x SD; ³ Cut-off at mean + 3 x SD; ⁴ Cut-off at 26 titer units (best discriminating level); ⁵ Cut-off at 15 titer units; ⁶ Cut-off set 0.05 above the value used by the authors (Mean + 3 x SD); ⁸ Cut-off set at 2.28 titer units; ⁹ Cut-off set at mean + 3 x SD, sera adsorbed with *M. phlei*; N.T. Not tested; * p<0.05, ^ p<0.02, **p<0.01 (Disease patients vs Healthy controls).

data show an unusually high coefficient of variation which together with a lower mean than the control group suggests that their Crohn's set might have a wide range of variation including many non-responders, rather than having a lower average level of response.

In the paper by Stainsby et al.¹⁵, no significant differences are found when comparing means. However, taking only the qualitative results, i.e., the frequencies of positive reactors, it appears to be quite unlikely that the distribution was the same in Crohn's (active and inactive) and controls. Their argument that a general non-specific reactivity against mycobacterial antigens exists in Crohn's disease patients, can be partially supported by other works which illustrate some degree of seroreactivity against yeast and bacterial antigens in Crohn's disease. However, there is also evidence, presented herein and elsewhere on reactivity of tuberculosis patients to *M. paratuberculosis* antigens, that cross-reactions to mycobacteria are common, and do not necessarily indicate that the individual is reacting to any antigen. Rather, the relatively low proportion of reactors among Crohn's disease patients can be interpreted as an argument against an increase in unspecific reactivity against digestive antigens.

In the study by Morgante et al.¹³ only a moderate increase in anti-mycobacterial reactivity was found among CD patients. Regarding reactivity against tuberculosis antigens no significant differences were observed in the average OD of Crohn's disease patients and Healthy controls (the p value in their table must be a mistake), although they had larger average OD. There was a significant difference regarding reactivity against Map antigens, both in absorbed and in non-absorbed tests. The differences were more marked when the results were considered as proportions of "positive" individuals, but then only the tuberculosis group significantly differed from the control group.

A significant difference in reactivity between the Crohn's disease and control groups was observed when all data were collectively analyzed (Tables 2 and 3). Table 2 displays the results in quantitative terms, showing that the overall increase in anti-mycobacterial reactivity in the CD groups is 30%. This difference gets even larger (58%) if only Map antigens are considered. Table 3 illustrates results comparing frequencies of "positives" in each individual report and collectively. In several of these reports, no significant differences were observed by the authors. Reanalysis and shifting of the ELISA cut-off values led to a significantly higher proportions of "positives" in the Crohn's disease group as compared to controls (Kobayasi et al.⁹ -p<0.05-, Tanaka et al.¹⁸ -p<0.05-, Brunello et al.² -p<0.05-, Stainsby et al.¹⁵ -p<0.05). Collectively, more than three times more positive reactors were observed among the Crohn's disease groups than in controls. The ulcerative colitis groups had approximately the same proportion as the control group, while the tuberculosis group had the highest proportion of positive reactors.

Our quantitative ELISA results for Crohn's patients are in the lower part of the range reported in previous studies.. Only the present study and that of Morgante et al.¹³ incorporated a tuberculosis positive control group with quantitative data (the data by Thayer et al.¹⁹ on PPD positive were not taken into account since in no other study the intradermal PPD status of normal controls was known). In both studies, the tuberculosis group had the highest reactivity against *M. paratuberculosis* antigens.

It is also very noticeable that there was a highly significant difference among the levels of antimycobacterial reactivity of American and European groups, no matter they were healthy controls, Crohn's disease, ulcerative colitis or tuberculosis patients. This particularly clear for healthy controls that are nearly four times more often positive to mycobacterial antigens in America than in Europe.

Very little more can be pointed out from the results presented here, since the variety of conditions under which each study was carried out is reflected in the lack of reproducible patterns in the results. For instance, while in the study by Stainsby et al.¹⁵ it seems that the highest proportion of reactors among CD patients is found against Map antigens (about three times more than *M. tuberculosis*), in the work by Morgante et al.¹³, the proportions do not practically differ. Overall, it seems that all populations seem to react less to Map antigens than to generic mycobacterial antigens, this loosely indicating that Map reactivity could be slightly more specific.

DISCUSSION

Our ELISA results showed that the highest reactivity was found in the tuberculosis group. This finding points out that the reactivity was not paratuberculosis specific. However, this same fact indicates that the ELISA test has a certain anti-mycobacterial antibodies specificity. Although that may be of low diagnostic interest, at the current state of knowledge on Crohn's disease, it could help support or rule out the mycobacterial hypothesis on the aetiology of this disease. Our results support this hypothesis since Crohn's disease patients ELISA values were not significantly different than those observed with a definite mycobacterial disease (tuberculosis), and significantly ($p < 0.01$) differed from both healthy controls and ulcerative colitis patients. This comparison to controls and ulcerative colitis patients is in agreement with the results of Thayer et al.¹⁹ and Wayne et al.²¹. However, it is known that humoral responses in paratuberculosis (and mycobacterioses in general) are dependant on the spectral stage of the disease and it is common to find infected individuals that fail to elicit humoral reactions against antigens of the causing agent. This kind of response is generally associated with paucibacillary types of infection which are characterized by few mycobacteria.

Another interesting observation is that HC have higher generic anti-mycobacterial antibody levels than UC. Although this finding should be confirmed in other studies and prove relevant in the future, currently the simplest explanation is to assume that being the UC groups smaller than any of the others, it is most likely that individual decreased immune response that could even be caused by treatment could bias the average in comparison with the HC which are larger groups. In fact, when only Map antigens are considered, the UC group becomes non significantly different from HC group.

Different and conflicting results between laboratories could be due to differences in antigen composition, laboratory methodology, and/or in environmental sensitization of Crohn's disease and control populations. Data from other studies in which no significant differences were found in the Crohn's disease groups, had rather high coefficients of variation. Perhaps the failure to find statistically significant differences could be due to an excess of variability. Pooling all available results suggests a strongly significant relationship, both in quantitative and in qualitative terms, that apparently overcomes the inherent variability of different conditions in which each study was performed. The difference between continents in mycobacterial reactivity is an unexpected observation that seems to represent more than an effect of individual studies variability and deserves further studies to clarify its meaning. Unfortunately, the information obtained in the present study do not allow more than to point out its existence.

The analysis of ELISA results presented here indicate that there is a worldwide, increased anti-mycobacterial IgG antibody level among Crohn's disease patients. This is not new, but its significance has been strengthened by the accumulation of data coming from different laboratories at different times, and using different mycobacterial antigens. This is not conclusive with regard of an etiological

demonstration but further supports the view that Crohn's disease might be related to Map infection. In our opinion, like the many human diseases for which an infectious aetiology has been demonstrated in the last years, the Koch postulates are not completely valid because infection is necessary, but no sufficient to cause disease. Even in the classical case of paratuberculosis, the pathogenesis paradigms need to be reviewed in the light of the unsuspected high prevalence of Map in animals recently uncovered by application of more efficient techniques compared to the relatively low immunological and clinical prevalence. This observation, together with the known variability in immune response and bacterial burden in spectral infections, will explain why Map is also present in healthy populations, and sometimes rare to detect in sick individuals. Under this hypothesis, It is important then to begin research focused on the most likely co-factors: the genetic, that has already been shown to play a role in Crohn's disease and in paratuberculosis, and the environmental, that has received much less attention. In this sense, the time and intensity of contact with this or other mycobacteria seem to be one of the most likely factors that can disturb the immune system enough as to allow Map to find its way to cause disease.

NOTE

The first version of this paper was circulated to the members of the International Association for Paratuberculosis in The Paratuberculosis Newsletter, 12(2):14-25. 2000.

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