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Mucosal cell-mediated immunity to mycobacterial, enterobacterial and other microbial antigens in inflammatory bowel disease

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SUMMARY

Culture studies have suggested that *Mycobacterium paratuberculosis* may play a role in the aetiology of Crohn's disease. However, evidence of sensitization to mycobacterial antigens amongst patients with Crohn's disease has not yet been adequately demonstrated. Previous studies of cell-mediated immunity (CMI) in Crohn's disease were restricted to responses of peripheral blood mononuclear cells (PBMC) to mycobacterial antigens. In this study we have investigated the proliferative responses of both PBMC and mesenteric lymph node mononuclear cells (MLNMC) to a range of mycobacterial and non-mycobacterial antigens. There was no evidence of specific sensitization in the responses of MLNMC and PBMC from patients with inflammatory bowel disease (IBD) to the mycobacterial antigens. However, anergy to *M. paratuberculosis* could not be excluded. IBD MLNMC responses to most antigens were generally greater than those of PBMC, which were often undetectable. When compared with controls, there was evidence of increased CMI to a range of non-mycobacterial antigens, especially *Yersinia enterocolitica*, amongst both MLNMC and PBMC from patients with Crohn's disease and ulcerative colitis (UC). These results do not provide support to the proposed role of mycobacteria in the pathogenesis of Crohn's disease, but indicate that further investigation may determine a role for bacterial-specific T cell-mediated responses in the pathogenesis of IBD.

Keywords Crohn's disease ulcerative colitis lymph node T lymphocytes bacterial antigens

INTRODUCTION

There is evidence of a genetic predisposition to both ulcerative colitis (UC) and Crohn's disease [1,2]. However, the lack of absolute concordance in identical twins and the relatively late onset of the disease in most individuals suggest that there is a significant environmental factor that is important in the aetiology.

Many infectious agents, including several species of enterobacteria, have been implicated in the aetiology of UC and Crohn's disease, but they have tended to be disregarded because of negative serological or culture studies [3–6]. *Escherichia coli* and *Yersinia enterocolitica* have attracted particular interest; *E. coli.* because of the presence in inflammatory bowel disease (IBD) sera of antibodies which cross-react between the Kunin antigen of *E. coli* and other enterobacteria, and an antigen derived from the human colon [7]; *Y. enterocolitica* because of certain similarities between yersiniosis and Crohn's disease [8]. Similar histological changes to those seen in Crohn's disease have been noted in the mucosa of patients with reactive arthritis, following infection with a variety of agents, particularly enterobacteria [9]. In more recent years the clinical and histological similarities of Crohn's disease to Johne's disease of ruminants [10], and the isolation of *Mycobacterium paratuberculosis* from patients with Crohn's disease, but not controls, produced a candidate organism for involvement in the aetiology of Crohn's disease [11,12].

Immunological support for this hypothesis has not been consistently demonstrated. Most of the studies in IBD have been of humoral immunity and have yielded conflicting results [13,14]. The immune response in mycobacterial infection, however, is primarily cell-mediated and the fact that Crohn's disease is a granulomatous disorder indicates that T cells may be involved in its pathogenesis. Studies relating to cell-mediated immunity (CMI) and mycobacteria in IBD have been restricted to investigations of peripheral blood mononuclear cell (PBMC) responses [15–17]. In IBD it is more appropriate to study CMI in gut-associated lymphoid tissues (GALT) [18], as this is where the most marked histological changes occur and also because of the distinct immunological functions of GALT. Our preliminary studies of CMI directed at mycobacterial antigens [19] demonstrate the importance of investigating CMI of GALT in addition to that in PBMC. This view is supported by observations made in other inflammatory disorders: in rheumatoid

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Table 1. Patient details

Group	*n*	M/F	Age (years) Median	Age (years) Range	Drug therapy
Control	21	6/16	62·5	19–85	nil
Ulcerative colitis	5	3/2	31	22–70	3/5 steroids
Active Crohn's disease	20	11/9	34	18–69	10/19 steroids
Inactive Crohn's disease	12	6/6	39	23–72	4/12 steroids

arthritis there are differences in the responsiveness of PBMC and synovial fluid MC (SFMC) to mycobacteria [20]. Additionally, SFMC responses to the causative organisms in reactive arthritis are considerably greater than those of PBMC [21,22].

We have therefore studied CMI responses to a panel of enteric and non-enteric agents in patients with IBD and control subjects. Lymphocyte proliferation assays were performed using mononuclear cells obtained from both the peripheral blood and mesenteric lymph nodes (MLNMC), the latter being a relatively easily obtained sample of GALT, that is not contaminated by luminal or mucosa-associated flora.

PATIENTS AND METHODS

Patients

Consecutive patients undergoing intestinal resection for ulcerative colitis ($n=5$), active ($n=20$) or inactive ($n=12$) Crohn's disease, at the General Hospital, Birmingham, were studied. The diagnosis of IBD was based on typical histological, radiological and clinical findings. Crohn's disease was considered to be active if there was evidence of raised C-reactive protein or alpha-1 acid glycoprotein, or a raised platelet count. Control subjects ($n=21$) were undergoing laparotomy for a variety of surgical procedures (cholecystectomy, resection for carcinoma of the colon, colectomy for diverticular disease and/or carcinoma, repair of an incisional hernia and haemicolectomy for a caecal volvulus). Patients with macroscopic intra-abdominal sepsis were excluded. Patient details and drug therapies are given in Table 1.

Isolation and culture of lymphocytes

Lymph nodes were collected into transport media (ice cold RPMI 1640 culture medium containing 2% heat inactivated, immunoglobulin depleted, bovine serum), minced aseptically and filtered to remove tissue debris. MLNMC were separated from this filtrate by density gradient centrifugation over Ficoll–Paque. PBMC were obtained similarly from defibrinated or heparinized peripheral blood. Cells were washed twice in phosphate-buffered saline (PBS) and resuspended in RPMI 1640 containing 10% heat-inactivated A^+ serum, 100 U/ml penicillin and gentamicin and supplemented with L-glutamine. The cells were counted, assayed for viability by Trypan blue exclusion and cultured in round-bottomed 96-well microtitre plates at 5×10^4 cells/well. The cells were cultured for 6 days in the presence of culture medium alone or with the antigens or mitogens described below. Tritiated thymidine (0·15 μCi) was added to each well 18 h before harvesting, after which [^{3}H]thymidine incorporation was measured using a liquid scintillation counter.

Antigens

The antigens used in these proliferations were obtained from a variety of sources: *M. paratuberculosis* from Dr. A. Nolan, Veterinary Laboratory, Weybridge, UK; *Mycobacterium avium-intracellulare* from Dr R. Wise, Dudley Road Hospital, Birmingham, UK; *Chlamydia trachomatis* and *Salmonella agona* from Drs J. Pearce and G. Clark, respectively, Department of Microbiology, University of Birmingham, UK; *Y. enterocolitica* 0·3 from Dr K. Granfors, Department of Medical Microbiology, University of Turku, Finland. All bacteria were either heat killed or γ-irradiated, checked for sterility, suspended in RPMI 1640 containing 10% human group A serum and stored at $-20°C$ before use. Other antigens used in these studies included *E. coli*, *Candida albicans*, influenza (H3 N2) and the recombinant 65-kD heat shock protein of *Mycobacterium leprae* (a gift from Dr M. J. Colston, National Institute for Medical Research, Mill Hill, UK). All antigens were used at previously determined optimum concentrations for proliferation studies.

Other reagents

Purified protein derivative (PPD) (Evans Medical, Dunstable, UK) of *Mycobacterium tuberculosis* was used at a previously determined optimum concentration of 20 μg/ml. As positive controls, phytohaemagglutinin (PHA) and recombinant IL-2 (rIL-2) (100 U/ml; Cetus Corporation, Emeryville, CA) were used to confirm viability and detect evidence of activation.

Statistical analysis

Differences between groups were compared using Mann–Witney *U*-tests, using two-tailed tests of significance.

RESULTS

Proliferative responses to IL-2 are shown in Fig. 1. Increased proliferation in response to IL-2 occurred in MLNMC and PBMC populations from both UC and active Crohn's disease. Responses in patients with inactive Crohn's disease were not significantly different from controls. Figures 2 and 3 and Table 2 summarize the proliferative responses of MLNMC and PBMC from all patients with Crohn's disease, UC and controls. There were no differences between responses obtained from patients with colonic carcinoma compared with other (non-malignant) controls. Proliferative responses of MLNMC were generally greater than those of autologous PBMC in active Crohn's disease and UC, except for three patients with active Crohn's disease where a reverse pattern was obtained.

In terms of T cell responses to mycobacterial antigens (Fig. 2), proliferations to *M. avium-intracellulare* and *M. paratuberculosis* were low in all groups. There was some evidence of

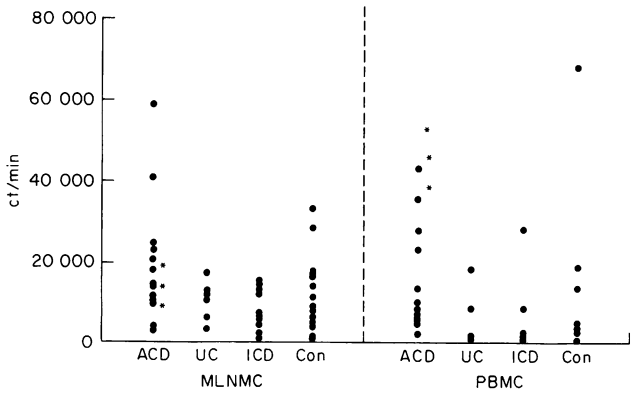

Fig. 1. Lymphoproliferative responses to IL-2 in patients with inflammatory bowel disease (IBD) and controls. Mesenteric lymph node mononuclear cells (MLNMC) and peripheral blood mononuclear cells (PBMC) (5×10^4/200 μl) from four disease groups (active Crohn's disease (ACD), inactive Crohn's disease (ICD), ulcerative colitis (UC) and controls (Con)) were cultured in the presence of IL-2 for 6 days. Proliferative responses were measured by uptake of [^{3}H]thymidine. *Three patients with active Crohn's disease with higher proliferative responses in PBMC than in MLNMC.

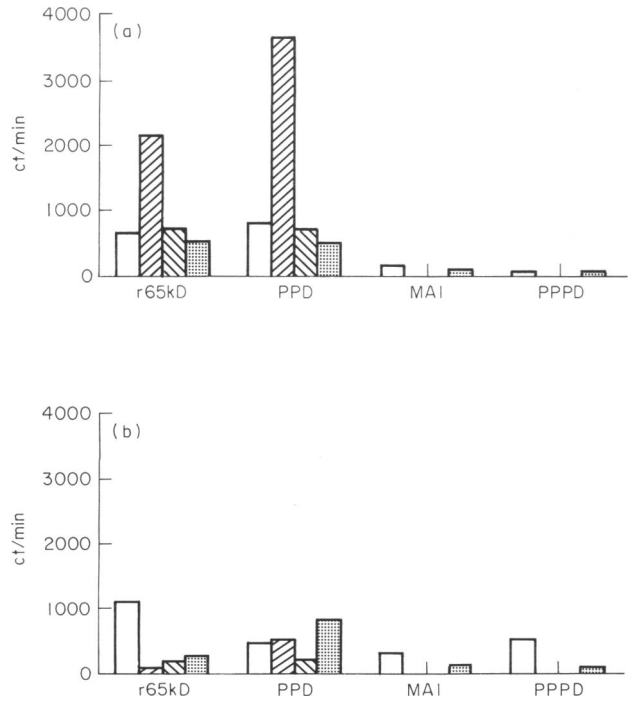

Fig. 1. Lymphoproliferative responses to IL-2 in patients with inflammatory bowel disease (IBD) and controls. Mesenteric lymph node responses to mycobacterial antigens. MLNMC and PBMC (5×10^4/200 μl) from patients with inflammatory bowel disease (IBD) and controls were cultured with purified protein derivative of *M. tuberculosis* (PPD), the recombinant heat shock protein of *M. leprae* (r65kD), *M. avium-intracellulare* (MAI) and a purified protein derivative of *M. paratuberculosis* (PPPD). Ranges are shown in Table 2. □, Crohn's disease; ■, ulcerative colitis; ▧, inactive Crohn's disease; ▨, controls.

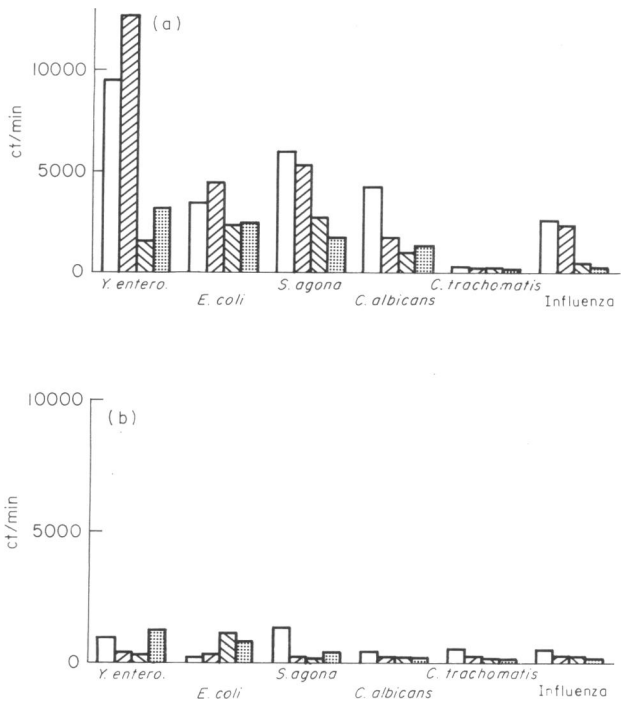

Fig. 3. Median mesenteric lymph node mononuclear cell (MLNMC) (a) and peripheral blood mononuclear cells (PBMC) (b) proliferative responses to non-mycobacterial antigens. MLNMC and PBMC were cultured for 6 days in the presence of a range of antigens and proliferation measured by [^{3}H]thymidine uptake. Ranges are shown in Table 2. Details as in Fig. 2.

increased proliferation in PBMC populations from patients with IBD compared with controls, but this was not observed with MLNMC and was very low in comparison with maximal responses observed. There was no evidence of increased proliferation in response to the PPD of *M. tuberculosis* and the recombinant heat shock protein of *M. leprae* in either MLNMC or PBMC populations from patients with active IBD.

Responses to *Chlamydia trachomatis*, included in the bank of antigens primarily as a negative control, were again similar in all groups. As would be expected, proliferation in response to this organism was insignificant, apart from in one patient with Crohn's disease. In this patient, however, the reponse to *C. trachomatis* was low in comparison with the maximal response observed. Stimulation with influenza and *C. albicans* gave rise to higher levels of proliferation, but this again appears to be a non-specific response. There were no significant differences between the patient groups despite the trend towards increased responsivity of MLNMC from patients with Crohn's disease.

There was also evidence of increased proliferation of MLNMC from patients with IBD to all the antigen preparations of enteric bacteria (Fig. 3). There was no difference between the responses of MLNMC from patients with active Crohn's disease to *E. coli* (median 4592 ct/min) and *S. agona* (median 5966 ct/min). The maximum proliferative response, however, was most often elicited by *Y. enterocolitica* (median 9511 ct/min) with response to this antigen being significantly greater than responses to *E. coli* ($P = 0{\cdot}049$), influenza, PPD, the recombinant heat shock protein (r65 kD), and *C. trachomatis*

Table 2. Lymphoproliferation in response to microbial antigens: medians and ranges (ct/min) in disease groups and controls

		MLNMC		PMNC	
		Median	Range	Median	Range
IL-2	Cont.	6337	(320–58 572)	5988	(186–68 435)
	ICD	6642	(783–15 186)	2339	(36–2814)
	UC	11 606	(3106–17 044)	4798	(818–18 157)
	ACD	10 895	(27 340–58 921)	7866	(1891–43 216)
r65kD	Cont.	528	(32–24 071)	283	(122–3172)
	ICD	727	(36–12 192)	190	(33–8825)
	UC	2131	(451–6053)	77	(18–10 048)
	ACD	637	(26–15 436)*	1105	(66–6058)
PPD	Cont.	497	(32–9828)	832	(37–14 772)
	ICD	703	(59–4425)	180	(28–2601)
	UC	3632	(572–14 975)	534	(42–19 575)
	ACD	810	(45–21 494)*	489	(118–24 267)
MAI	Cont.	95	(26–4202)	148	(37–3567)
	ICD	NT		NT	
	UC	NT		NT	
	ACD	171	(33–3856)	326	(57–14 259)
PPPD	Cont.	54	(19–20 102)	87	(48–832)
	ICD	NT		NT	
	UC	NT		NT	
	ACD	45	(32–5231)	531	(55–9136)
E. coli	Cont.	2396	(20–10 058	794	(63–8652)
	ICD	2318	(83–8800)	1108	(70–21 050)
	UC	4410	(273–16 181)	277	(63–14 965)
	ACD	3374	(57–37 385)†	198	(158–2041)
Y. enterocolitica	Cont.	3212	(38–16 519)	1189	(62–12 923)
	ICD	1493	(76–9348)	290	(42–8729)
	UC	12 738	(546–26 117)	406	(127–19 702)
	ACD	9511	(37–31 362)	902	(109–31 203)
S. agona	Cont.	1623	(64–16 290)	318	(45–4482)
	ICD	2661	(54–18 112)	189	(35–5323)
	UC	5252	(1039–15 345)	194	(87–10 652)
	ACD	5966	(33–23 811)‡	1300	(71–32 916)
C. albicans	Cont.	1305	(22–34 009)	187	(43–10 989)
	ICD	931	(44–27 095)	165	(34–10 513)
	UC	1715	(449–13 943)	226	(155–25 354)
	ACD	4217	(30–34 840)§	417	(45–44 953)
C. trachomatis	Cont.	128	(33–3882)	128	(72–1073)
	ICD	188	(10–525)	137	(80–1362)
	UC	194	(71–718)	249	(53–6023)
	ACD	225	(32–12 453)*	437	(90–13 456)
Influenza	Cont.	253	(25–7764)	105	(44–6934)
	ICD	438	(60–9905)	221	(32–849)
	UC	2266	(390–14 259)	212	(166–9560)
	ACD	2532	(29–10 182)*	463	(25–12 091)

*$P < 0{\cdot}001$. †$P = 0{\cdot}049$ compared with proliferations to *Y. enterocolitica*.

‡$P = 0{\cdot}058$. §$P = 0{\cdot}13$ compared with proliferations to *Y. enterocolitica*.

ICD, Inactive Crohn's disease; UC, alterative colitis; ACD, active Crohn's disease; PPD, purified protein derivative; r65kD, recombinant heat shock protein; MAI, *M. avium-intracellulare*; PPPD, purified protein derivative of *M. paratuberculosis*.

($P < 0{\cdot}001$ in all cases). Although not reaching statistical significance, responses to *Y. enterocolitica* were also raised compared with *S. agona* ($P = 0{\cdot}059$), and *C. albicans* ($P = 0{\cdot}3$). A similar pattern was observed with responses in patients with UC, whereas responses to these organisms in patients with inactive Crohn's disease and controls were low and all of the same order of magnitude.

DISCUSSION

The data presented here show that both PBMC and MLNMC proliferate in response to a range of different antigenic stimuli. Responses in patients with IBD are generally easier to detect in MLNMC than in autologous PBMC. This may be due to differences in the phenotype of the populations studied, or their activation state. This difference between MLNMC and PBMC responses emphasizes the importance of examining lymphocyte populations closer to the site of inflammation than those in peripheral blood. The relevance of this approach has already been demonstrated in studies of synovial fluid (SFMC) and PBMC responses in reactive arthritis (ReA) [21,22]. Although the highest responses in ReA occur in SFMC stimulated with the causative organisms, SFMC also recognize a variety of organisms. This may be due to cross-reactivity with the causative organism or may represent response to antigens for which the patient has T cell memory. In addition, it has been demonstrated in ReA that at least some of the heightened responsiveness of SFMC may be due to an increased ability of SF antigen-presenting cells to support T cell proliferative responses [23]. Our results suggest that similar phenomena may be occurring in lymph nodes from patients with IBD. A similar pattern of increased responsiveness in comparison with PBMC has been demonstrated with gut mucosal lymphocytes stimulated with *Bacteroides* and *Staphylococcus aureus* [18]. This provides further evidence for the localization in areas of intestinal inflammation of lymphocytes which have became sensitized to the gut flora.

In contrast to patients with IBD, several controls failed to respond to any bacteria, using either MLNMC or PBMC, with levels of [^{3}H]thymidine uptake often being barely detectable. There is increased responsivity to IL-2 amongst mononuclear cells from patients with IBD compared with controls. This contradicts a report of diminished responses to IL-2 in patients with Crohn's disease [24]; this study examined solely PBMC responses, and again demonstrates the need to examine mononuclear cell populations other than solely those of peripheral blood when carrying out immunological studies of IBD.

This difference in responses between patient and controls was greater in MLNMC. In both Crohn's disease and UC, MLNMC were more responsive to IL-2 than were PBMC. This may represent increased activation *in vivo* and the subsequent acquisition of IL-2 receptors. It is already recognized that there are increased numbers of cells bearing early markers of activation in Crohn's disease [25].

The inclusion of *C. trachomatis* in the bank of antigens used in this study was primarily as a negative control. Few individuals would be expected to have become sensitized to this organism, so proliferation of mononuclear cells in response to it should be low. However, the suggestion that *C. trachomatis* may play a role in the pathogenesis of Crohn's disease has recently been resurrected [26]. Consequently, it was of interest that proliferative responses to this organism, both in patients with IBD and controls, were indeed low, providing evidence against the involvement of *C. trachomatis* in the pathogenesis of IBD.

Responses of MLNMC and PBMC from patients with IBD to mycobacterial antigens fail to provide any conclusive evidence of specific CMI to *M. paratuberculosis* in Crohn's disease or UC. Despite a generalized increase in responsiveness, particularly in MLNMC in active IBD, responses to the PPD of *M. tuberculosis* and r65 kD heat shock proteins (HSP) are low and parallel the degree of activation of the cells as measured by responsiveness to IL-2. This contradicts the observations of other workers [16] but is supported by the negative findings of macrophage inhibition factor assays on PBMC [17]. The maximal responses of both MLNMC and PBMC from patients with IBD and controls were to enterobacterial antigens and considerably higher than those seen to mycobacterial antigens.

The 65-kD heat shock protein of *M. tuberculosis* and *M. leprae* has been well characterized, is highly conserved between bacterial species and is a strong immunogen in mycobacterial infection [27]. The lack of evidence for increased sensitization to this antigen in patients with Crohn's disease again does not support a role for *M. paratuberculosis* in the aetiology of Crohn's disease.

The fact that the 65-kD protein of mycobacteria is cross-reactive with other bacterial species such as *E. coli* suggests that proliferation in response to this protein should correlate with that to other antigen preparations used in our study. This proved not to be the case and suggests that the 65-kD antigen does not play a significant role in the immunopathology of IBD. However, a report of experimentally induced chlamydial hypersensitivity employing a genus-specific HSP [28] supports a possible role for T cell recognition of organism-specific epitopes of HSPs in the pathogenesis of bacterial inflammatory diseases.

The responses to *M. avium-intracellulare* and the PPD of *M. paratuberculosis* are of more interest. It was difficult to detect any proliferative response to these antigens in MLNMC of IBD patients, despite producing high proliferation in one control patient. There was, however, evidence of increased activity in the peripheral blood of these patients, albeit at a low level. Thus, if *M. paratuberculosis* is playing a significant role in IBD one would have to postulate that it was inducing antigen-specific suppression within the mucosal lymphoid system. Evidence for mycobacterial induced suppressor cell activity has recently been sought in PBMC [29], but unfortunately it is not clear if the suppressor cell activity induced was *M. paratuberculosis*-specific or not.

The most striking finding in our study was the marked increase in responsivity of MLNMC from patients with IBD to *Y. enterocolitica*. Given the high degrees of cross-reactivity between enterobacterial species, the consistently raised levels of proliferation to *Y. enterocolitica* in comparison with those to *S. agona* and *E. coli* was an unexpected finding. *Y. enterocolitica* causes an acute enterocolitis which may share similarities, in terms of histology and tropism for the terminal ileum, with Crohn's disease [30]. Although it has previously been accepted that acute Yersiniosis never progresses to Crohn's disease [8], the recent recognition of a chronic form of yersiniosis [31] is of interest. In patients with chronic disease, *Y. enterocolitica* could not be cultured from faeces or diseased mucosa, despite the employment of specific culture techniques for this organism.

In addition, standard agglutination tests used to detect

antibodies to the organism are often negative in the chronic stages of the disease. Previous studies, which have failed to demonstrate serological evidence of yersinial involvement in Crohn's disease [32,33], employed such agglutination methods so it is of particular interest that we and other workers, using different techniques, have shown elevated serum antibody levels to *Y. enterocolitica* and other enterobacterial species in active Crohn's disease [5,34]. This observation has been explained as being due to cross-reactivity between *Y. enterocolitica* and commensal enterobacteria to which the patient is normally exposed. Our results, however, suggest that against a background of increased CMI to a range of enteric organisms, there may be specific CMI in IBD directed at *Y. enterocolitica*.

One of the histological features in a large proportion of patients with Crohn's disease is the presence of granulomata. This is characteristic of infections where the causative organisms are capable of existing intracellularly and is a feature of mycobacterial disease. The possible role for mycobacteria in Crohn's disease is well documented [35] but this study has failed to provide support for the involvement of mycobacteria in the pathogenesis of IBD. *Y. enterocolitica* invades via mucosal interaction and can also exist intracellularly [36] and at least one species of Yersinia, *Y. pseudotuberculosis*, initiates a granulomatous disease of the terminal ileum [37]. Further characterization of the Yersinial antigens stimulating the proliferative responses reported here is required before a role for the organism in the etiopathogenesis of IBD can be proposed.

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