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Peripheral cell-mediated immune response to mycobacterial antigens in inflammatory bowel disease

D. S. ROWBOTHAM, P. D. HOWDLE & L. K. TREJDOSIEWICZ *Division of Medicine, Research School of Medicine, St James's University Hospital, University of Leeds, Leeds, UK*

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SUMMARY

A mycobacterial etiology has been proposed in Crohn's disease (CD). We have sought evidence of increased or modified T lymphocyte immune responses to *Mycobacterium tuberculosis* and *Myco. paratuberculosis* in patients with CD ($n = 13$), compared with ulcerative colitis (UC; $n = 17$) and controls ($n = 17$). Peripheral blood cells were cultured with phytohaemagglutinin (positive mitogen control), mycobacterial purified protein derivative (PPD) preparations, lysates, column fractions and whole, heat-killed bacteria. Responses of T cells and T cell subsets were assessed by expression of activation markers (CD25, CD69), coupled with blastogenesis assays (^{3}H-thymidine uptake) and estimates of proliferation. Virtually all patients responded to *Myco. paratuberculosis* and *Myco. tuberculosis* antigens. There were no significant differences between patient groups, although there was a very high overall correlation ($r = 0{\cdot}95$; $P < 0{\cdot}0001$) between responses to the two mycobacterial species. Most of the activation and proliferative responses resided in the $CD4^+$ (T helper) subset. Although up to 15% of $CD8^+$ (suppressor/cytotoxic) cells also became activated, the $CD8^+$ cells did not proliferate subsequently. Cells expressing the alternate $\gamma\delta$ form of the T cell receptor (TCR $\gamma\delta^+$) did not activate or proliferate in response to mycobacterial antigens. There were no differences in any of these parameters between patient groups. We conclude that there is no specific increase or alteration in cell-mediated anti-mycobacterial immunity in inflammatory bowel disease (IBD). Thus our data do not support a mycobacterial etiopathology of Crohn's disease.

Keywords Crohn's disease ulcerative colitis mycobacterial antigens T cell activation

INTRODUCTION

Many microbial agents have been implicated in the etiology of ulcerative colitis (UC) and Crohn's disease (CD), but most have been disregarded in view of negative serological studies or unsuccessful attempts at culture from intestinal tissue of affected individuals [1–4]. However, it has been found that sera of patients with inflammatory bowel disease (IBD) contain antibodies which cross-react between a colonic autoantigen and the Kunin antigen of enterobacteria including *Escherichia coli* [5]. *Yersinia enterocolitica* has also been implicated because of similarities between yersiniosis and CD [6]. Histological changes in the intestine very similar to CD have also been reported in patients with seronegative reactive arthropathy following infection with a number of organisms [7].

Infectious agents in UC remain unsubstantiated. CD, however, is the focus of major current debate. In recent years, attention has been focused on mycobacteriae as having a potential etiological role. It has long been noted that there are close similarities of CD with Johne's disease, a chronic granulomatous enteritis of ruminants [8] and other animals [9] caused by *Mycobacterium paratuberculosis*. The isolation and culture of *Myco. paratuberculosis* from patients with CD [10] sparked renewed interest in this organism in relation to CD. However, detection of mycobacterial DNA in CD tissue by amplification using the polymerase chain reaction (PCR) has produced conflicting results [11–17].

Immunological data relating to the mycobacterial theory of CD have also been conflicting. Positive associations have not been demonstrated consistently using the humoral antibody response [18–22]. Probably of greater importance are cell-mediated immune responses, as these are thought to be crucial to effective anti-mycobacterial immunity. However, varying results have been reported in studies of peripheral blood and mesenteric lymph node mononuclear cell responses [23–26]. In view of this lack of consensus the principal aims of this study were to test the hypothesis that cell-mediated immunity to mycobacterial antigens, particularly *Myco. paratuberculosis*, is increased in CD compared with UC and non-inflammatory bowel disease controls. A secondary aim was to examine the

Correspondence: L. K. Trejdosiewicz, Research School of Medicine, CSB Level 7, St James's University Hospital, Leeds LS9 7TF, UK.

responses of T cell subsets and the minority T cell population expressing the γ/δ heterodimer of the T cell antigen receptor (TCR), as TCR $\gamma\delta^+$ cells have been implicated in 'first line' immune defences against mycobacteria [27–30].

PATIENTS AND METHODS

Patients

In total, 30 patients with IBD were studied, diagnosed by standard clinical, radiological and histological criteria. Seventeen control non-IBD subjects were recruited from the hospital clinical and research staff. All subjects were questioned to ascertain their 'mycobacterial' history involving BCG inoculation, positive Heaf/Mantoux test or previous active tuberculosis. Information was also gathered regarding relevant drug therapy including steroids and other immunosuppressive agents. Details of all subjects used in this study are given in Table 1. Local ethical committee approval was obtained before commencement of the study.

Separation of peripheral blood lymphocytes

Mononuclear cells were isolated from heparinized peripheral venous blood by gelatin sedimentation at 37°C followed by density barrier centrifugation over Lymphoprep separation medium (Nycomed, Birmingham, UK). Interface cells were washed three times in RPMI 1640 buffered with 10 mM HEPES (1640/H), counted in trypan blue and suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (1640/FBS; ICN Pharmaceuticals, Thame, UK) at 2×10^6/ml. Peripheral blood lymphocyte (PBL) suspensions were $\geqslant 98\%$ viable and comprised 70–80% $CD3^+$ cells, as judged by flow cytometry (below).

Mycobacterial antigens

For routine experiments, mycobacterial purified protein derivative (PPD) preparations of *Myco. tuberculosis* and *Myco. paratuberculosis* were obtained from Evans Medical Ltd (Horsham, UK; 'Tuberculin' PPD) and the Central Veterinary Laboratories (Weybridge, UK; 'Johnin' PPD), respectively. PPD preparations were dialysed *versus* Dulbecco's PBS (PBSa) to remove preservatives, sterile 0·22-μm membrane filtered, and used in lymphocyte cultures at a previously pre-titred concentration of 5 μg/ml.

Table 1. Patient details

	Crohn's disease	Ulcerative colitis	Controls
n	13	17	17
Age: mean (years)	47·3	46·6	37·4
range	26–78	25–71	24–72
Sex ratio (male : female)	6 : 7	8 : 9	12 : 5
Immunosuppression (local/oral/parenteral)	10	12	0
BCG vaccination	6	8	14
Heaf positive	0	0	1
Previous/current TB	0	0	0

In order to investigate the effects of different forms of mycobacterial antigen preparations, *Myco. avium*, *Myco. bovis* (strain BCG-Pasteur), *Myco. paratuberculosis* and *Myco. tuberculosis* were cultured in a Category 3 room using Kirchner's medium (Southern Group Laboratory Ltd, Corby, UK) enriched with mycobactin for *Myco. paratuberculosis* [31]. Organisms were washed three times in Dulbecco's PBS with Ca^{2+} and Mg^{2+} salts (PBSc) and heat-killed at 70°C for 30 min. Either whole organisms were used or crude bacterial lysates were prepared by 2 min intermittent sonication and clarified by centrifugation at 100 000 ***g*** for 75 min at 4°C. Finally, for some experiments, mycobacterial sonicates and PPD preparations were fractionated on a Sephadex G50 column in 0·2 M NH_4Cl as a volatile buffer. Column fractions were pooled into >5 kD and 1–5 kD fractions and freeze-dried to completion before reconstitution in culture medium and filter sterilization (0·22 μm).

Lymphocyte stimulation assays

PBL cultures were established in replicate cultures in 200 μl final volumes in flat-bottomed 96-well tissue culture plates (ICN Pharmaceuticals) containing 2×10^5 responder cells per well, a concentration determined as optimal in preliminary experiments. Control cultures contained no antigen (negative controls) or phytohaemagglutinin (PHA; Wellcome Diagnostics, Dartford, UK) at a pre-titred optimal concentration of 2·5 μg/ml (positive controls). Cultures were maintained in a humidified 37°C incubator in 5% CO_2 for the times indicated.

Cell activation

T cell activation was assessed by 'two-colour' analytical flow cytometry using a FACScan instrument and Lysis II software (Becton Dickinson, Oxford, UK). Cells from individual wells were harvested after various time periods, and washed once in PBSa containing 1% w : v bovine serum albumin and 0·1% v : v NaN_3 (PBSa/BSA). Cells were labelled with PE-conjugated anti-CD2 (Dako, High Wycombe, UK), rather than CD3, as the latter is down-regulated on fully activated T cells. Simultaneously, T cell activation was assessed by expression of the early activation antigen, CD69, and the low-affinity IL-2 receptor, CD25, using appropriate FITC-conjugated antibodies from Dako and Becton Dickinson, respectively. All antibodies were used at pre-titred concentrations. After incubation, cells were washed once in PBSa/BSA, resuspended in 0·5 ml PBSa/BSA and analysed immediately. Acquisition parameters were established on the basis of tubes incubated with negative control (irrelevant) antibody PE and FITC conjugates (Dako). A 'live gate' based on size and granularity was used to exclude cell debris and platelets during acquisition. For analysis, a gate was drawn around the $CD2^+$ cells to exclude non-T cells. All results were compared against identical initial aliquots of 2×10^5 cells taken from the freshly separated PBL analysed at day 0.

PBL mitogenic responses

Triplicate cultures of PBL were pulsed with 0·5 μCi ^{3}H-thymidine (^{3}H-TdR) after various periods of time in culture, harvested after a further 18 h incubation onto glassfibre filters and then counted by liquid scintillation spectrometry. Results were expressed as stimulation indices (SI), where SI = (ct/min test)/(ct/min negative control) after background subtraction.

T cell proliferation

To assess proliferation by T cell subsets and subtypes, aliquots of PBL were cultured for 8 days and immunolabelled with anti-CD4, anti-CD8 (Dako) and TCSδ1 (anti-TCRVδ1; T Cell Diagnostics, Eurogenetics, Wokingham, UK) antibodies conjugated to PE, together with anti-CD3/FITC conjugate (Dako), suspended in exactly 0·5 ml PBSa/BSA and analysed by flow cytometry as above, except that acquisition was halted automatically the moment 10 000 lymphocytes had been counted and the remaining cell suspension volume measured to obtain an estimate of absolute cell numbers per tube.

Statistical analysis

Parametric statistics (mean ± s.d.) were used for descriptive purposes and a non-parametric test (Wilcoxon–Mann–Whitney *U*-test) was used to compare patient groups. Spearman rank and Pearson linear correlation coefficients were also calculated. All statistics were performed using Astute software (DDU Software, University of Leeds, Leeds, UK).

RESULTS

T cell responses to PHA

By criteria of up-regulation of CD69, CD25 and ^{3}H-TdR incorporation, PBL responses to PHA were consistently high in both patient and control groups, indicating good responder lymphocyte viability and normal function. SIs were in the range of SI = 20–60. There was no correlation between PHA-induced PBL response and the severity of disease inflammation or the use of immunosuppressive drugs.

T cell responses to mycobacterial antigens

The kinetics of T cell responses to mycobacterial antigens was delayed compared with PHA responses, as expected. For example, CD25 up-regulation reached maximal expression at 72–96 h in response to PHA. By contrast, up-regulation of CD25 expression in response to mycobacterial antigens did not occur significantly until 120 h, peaking at 144 h. Similar response kinetics was found with all the mycobacterial preparations tested and occurred in all patient groups (Fig. 1). Similarly, peak ^{3}H-TdR uptake and cellular proliferation in response to mycobacterial antigens were delayed compared with PHA. There was good overall concordance between the activation and proliferative responses of PBL to the mycobacterial antigens used in this study.

All mycobacterial preparations elicited T cell responses in all control subjects and in most patients. There was no correlation between immunosuppressive treatment and prior BCG vaccination or Mantoux positivity with *in vitro* responses to mycobacterial antigens. Although no significant differences between mycobacterial species could be determined (see below), there were some differences in the maximal responsiveness according to the type of antigen preparation. PPD preparations were the most stimulatory, followed by crude lysates and column fractions, whereas whole bacterial suspensions, although still capable of eliciting good responses, were less effective than the soluble preparations. For the sake of simplicity, the results described below were obtained with the PPD preparations.

PBL responses to Tuberculin and Johnin PPD. Although all normal subjects and most IBD patients showed positive responses to Tuberculin PPD, there was considerable variability

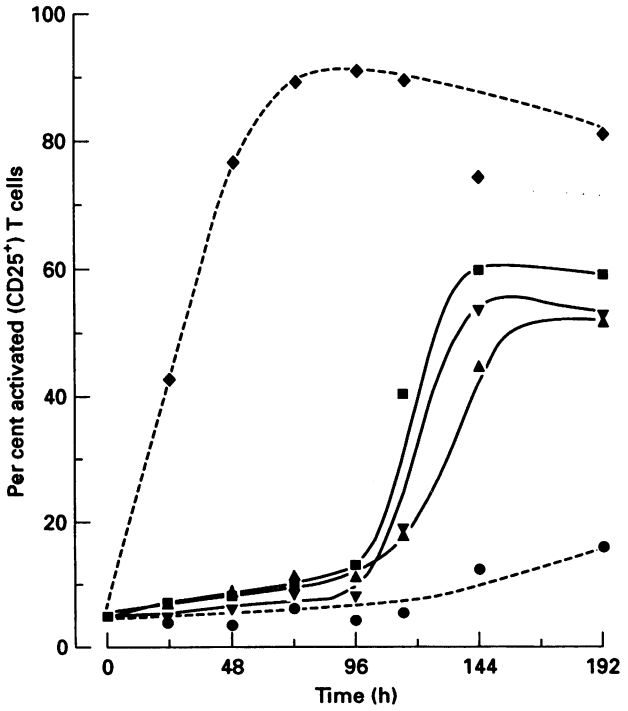

Fig. 1. Kinetics of T cell activation in response to mycobacterial antigens. Cell surface expression of CD25 by the CD2$^+$ subset of cultured peripheral blood lymphocytes (PBL) was assessed by flow cytometry. Cells were cultured with Tuberculin purified protein derivative (PPD) (■), Johnin PPD (▲), ultracentrifuged sonicate of *Mycobacterium tuberculosis* (▼) and phytohaemagglutinin (PHA) (◆) as a positive control. ●, Negative controls (no antigen). Data from one representative experiment.

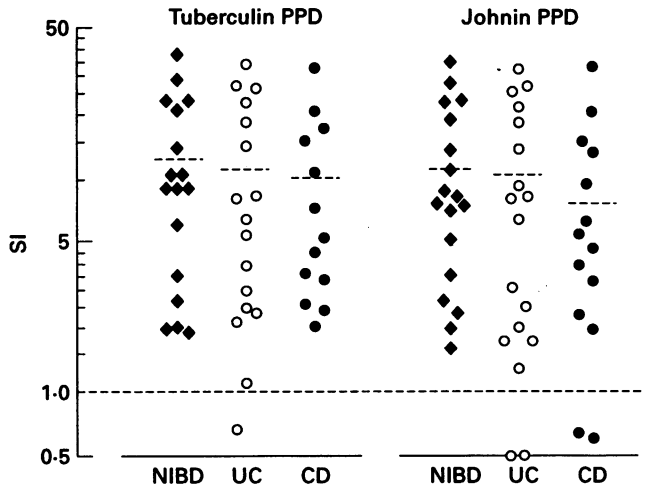

Fig. 2. Lymphocyte blastogenesis responses to mycobacterial purified protein derivative (PPD) preparations from *Mycobacterium tuberculosis* (Tuberculin) and *Myco. paratuberculosis* (Johnin) in patients with Crohn's disease (CD), ulcerative colitis (UC) and non-inflammatory bowel disease controls (NIBD). Ratios of ^{3}H-thymidine incorporation of test/control cultures are plotted as stimulation indices (SI); dashed lines represent sample means. There were no significant differences between any of the groups.

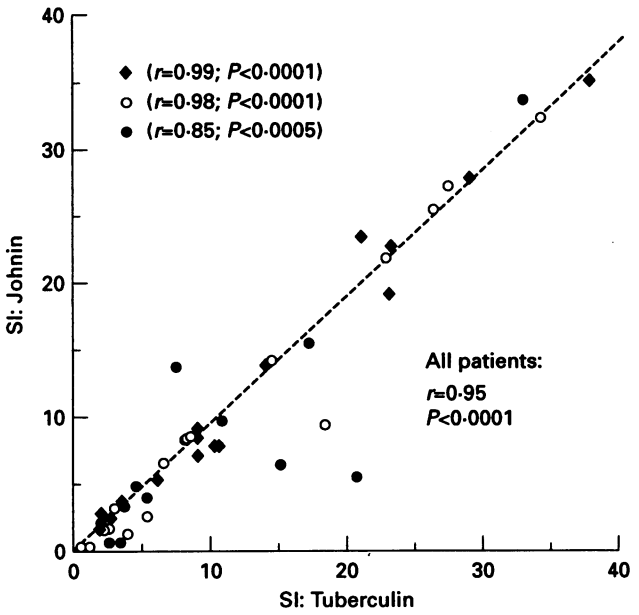

Fig. 3. Correlation between lymphocyte blastogenic responses to *Mycobacterium tuberculosis* (Tuberculin) and *Myco. paratuberculosis* (Johnin) purified protein derivative (PPD) preparations in control patients (NIBD) (◆) and patients with ulcerative colitis (UC) (○) and Crohn's disease (CD) (●). Pearson correlation coefficients (r) are indicated and were highly significant, indicating extensive recognition of cross-reactive mycobacterial antigens in all three patient groups. SI, Stimulation index.

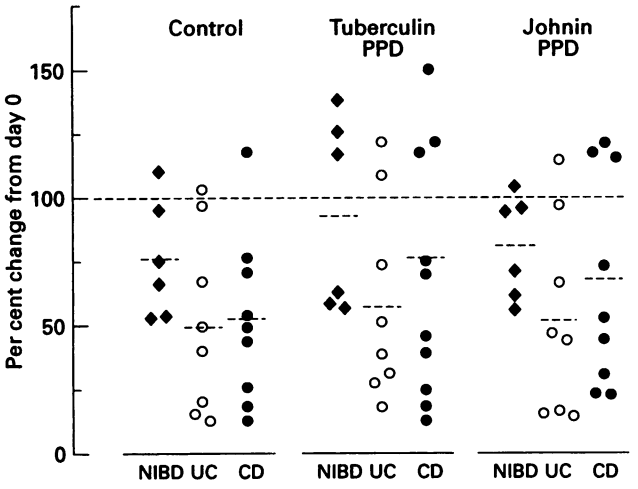

Fig. 4. Changes in absolute numbers of TCR $\gamma\delta^+$ T lymphocytes following 8 days culture of unseparated peripheral blood lymphocytes (PBL) with mycobacterial antigens from *Mycobacterium tuberculosis* (Tuberculin purified protein derivative (PPD)) and *Myco. paratuberculosis* (Johnin PPD), relative to day 0. TCR $\gamma\delta^+$ cells were enumerated by flow cytometry. Overall, there was no proliferative response by TCR $\gamma\delta^+$ cells, and there were no statistically significant differences between antigenic preparations or patient groups. NIBD, Non-inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease.

in the magnitudes of the responses in the ^{3}H-TdR stimulation assays (Fig. 2). However, the mean SI values from each group showed no statistically significant difference. Notably, there was no evidence of increased activation of proliferation of PBL towards Tuberculin PPD in subjects with CD compared with the other patient groups.

Very similar results were obtained with Johnin PPD. A similar individual variability was noted, but with no statistical difference between patient groups (Fig. 2). Subjects with CD did not display a preferential increase in immunity towards the PPD of *Myco. paratuberculosis* compared with the other patient groups. Nor did they display any increased immunity towards *Myco. paratuberculosis* compared with their response towards other mycobacterial species.

Comparison of PBL responses to mycobacterial PPD. In any given subject, whatever the intensity of the cell-mediated immune response to mycobacterial antigen (PPD), there was a very highly significant correlation between the responses observed after culture with *Myco. tuberculosis* PPD (Tuberculin) and *Myco. paratuberculosis* PPD (Johnin). This was observed in all three patient groups (Fig. 3). The correlation coefficients (Pearson r and Spearman rank r_s, respectively) were $r = 0{\cdot}85$, $r_s = 0{\cdot}84$ ($P < 0{\cdot}005$) in patients with CD; $r = 0{\cdot}98$, $r_s = 0{\cdot}97$ ($P < 0{\cdot}0001$) in patients with UC; and $r = 0{\cdot}99$, $r_s = 0{\cdot}97$ ($P < 0{\cdot}0001$) in the non-IBD controls. Results from the overall study population taken as a whole produced correlation coefficients of $r = 0{\cdot}95$, $r_s = 0{\cdot}93$ ($P < 0{\cdot}0001$).

Phenotypes of Mycobacterial-responsive PBL. Phenotypic analysis of the activated and proliferative T lymphocyte subtypes revealed that the response to mycobacterial antigens *in vitro* resided virtually exclusively in the majority TCR $\alpha\beta^+$ T cell subtype. By contrast, TCR $\gamma\delta^+$ did not show up-regulation of the CD69 and CD25 activation markers in any of the subjects tested. TCR $\gamma\delta^+$ cells did not proliferate in culture; rather, the absolute numbers of TCR $\gamma\delta^+$ cells tended to decline over the 8-day culture period, presumably due to death of unstimulated cells (Fig. 4). There were no significant differences between any of the patient groups or in responses to Tuberculin or Johnin preparations.

Further analysis of the TCR $\alpha\beta^+$ responding cell population demonstrated that the response resided mainly within the CD4$^+$ (T helper) subset. Up to 75% of CD4$^+$ cells became stimulated with up-regulation of expression of surface activation markers (CD25), and CD4$^+$ cells subsequently underwent a vigorous proliferative response. Although as many as 25% of the CD8$^+$ (suppressor/cytotoxic) subset also became activated in response to Tuberculin PPD, the CD8$^+$ population did not subsequently proliferate (Fig. 5). These observations were very comparable between the three patient groups, and there were no statistically significant differences in any of the parameters.

DISCUSSION

In addition to characteristic granulomatous histology of CD, there is considerable other evidence for a cell-mediated immunopathology in this condition [32]. Although mycobacteriae are a classical cause of granulomata, other microorganisms can also cause granulomatous reactions in man. An example would be *Y. pseudotuberculosis* [33], and one group has reported increased cell-mediated immune responses to a related species, *Y. enterocolitica*, in patients with IBD [26]. However, most interest has focused on mycobacteriae, particularly *Myco. paratuberculosis*. Mycobacterial infections are known to elicit

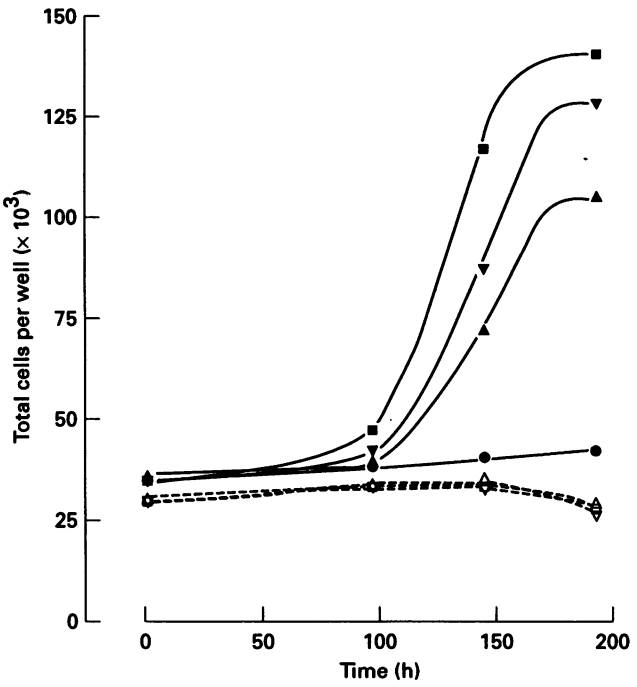

Fig. 5. Proliferative responses of lymphocytes to mycobacterial antigens. Absolute numbers of $CD4^+$ (closed symbols) and $CD8^+$ (open symbols, dashed lines) cells were determined by flow cytometry. Responses to purified protein derivative (PPD) preparations of *Mycobacterium tuberculosis* (squares), *Myco. paratuberculosis* (triangles) and clarified sonicate of *Myco. tuberculosis* (inverted triangles) are illustrated in this representative experiment. $CD8^+$ cells failed to proliferate in response to mycobacterial antigens in any of the patient groups, although both $CD4^+$ and $CD8^+$ cells showed vigorous proliferative responses to phytohaemagglutinin (PHA) (data not plotted for the sake of clarity).

strong T cell responses which cross-react with other mycobacterial species [34]. Thus, to support the *Myco. paratuberculosis* hypothesis, patients with CD would be expected to have enhanced T cell-mediated responses to the organism itself, with a high probability of increased cross-reactivity with other mycobacterial species.

Overall, the literature has failed to demonstrate consistent and definitive evidence of increased immune response to mycobacteriae in general, and to *Myco. paratuberculosis* in particular, in CD. The reported findings of raised anti-mycobacterial antibody levels in some studies [18,22] may merely represent previous exposure to other non-pathogenic species of mycobacteria which are ubiquitous in the environment, since it is clear, both from these data and other work, that mycobacterial species share common antigenic epitopes [34,35]. Indeed, in this study we have demonstrated a very high correlation between T cell proliferative responses towards the PPD preparations of *Myco. tuberculosis* (Tuberculin) and *Myco. paratuberculosis* (Johnin). Therefore it seems clear that human exposure to mycobacterial antigens in the form of environmental mycobacteriae, whether pathogenic or not, or immunization with BCG or any other mycobacterial vaccine, results in significant acquired immunity against a variety of mycobacterial species. It is interesting to note that in all three patient groups, there was no correlation between either age (and thus cumulative exposure to environmental strains of mycobacteriae) or BCG vaccination and T cell responses against the mycobacterial antigens tested. None of the patients had any history of previous or active tuberculosis, which might have acted as a confounding factor.

Our data show no evidence of increased cell-mediated immunity to either *Myco. paratuberculosis*, *Myco. tuberculosis* or indeed the widespread *Myco. avium* in patients with UC or CD. There was little or no overall difference in reactivity patterns between IBD patients and controls, and all three groups showed equally diverse responses. Thus, our work confirms and extends previous observations relating to cell-mediated immunity to *Myco. paratuberculosis* in IBD [25,26]. It has been argued that mycobacterial 'infection' in CD could be secondary to mucosal suppression of immunity, although this point of view is difficult to reconcile with the known increased mucosal immunoactivation and transmural inflammation [32]. Nevertheless, it is possible that altered anti-mycobacterial immunity in CD is manifest in a more subtle way than measured by crude lymphocyte blastogenesis assays. For this reason, we have also studied T cell activation and proliferation responses in the major T cell subsets (i.e. $CD4^+$ helper cells *versus* $CD8^+$ suppressor/cytotoxic cells) and subtypes (i.e. T cells expressing the $\alpha\beta$ *versus* $\gamma\delta$ forms of the T cell antigen receptor). Our observations that mycobacterial response is mainly a function of the TCR $\alpha\beta^+$ $CD4^+$ T helper cell subset in normal subjects agrees with previous observations [35,36]. We could find no evidence for differential responses in either subset between patient groups. Furthermore, there was no preferential activation of $CD8^+$ cells in patients with CD, and $CD8^+$ did not proliferate in response to mycobacterial antigens. Thus, it seems unlikely that specific suppressive responses mediated by $CD8^+$ cells are preferentially generated in patients with CD.

Another possibility is that T cells expressing the $\gamma\delta$ form of the TCR respond differentially to mycobacterial antigens in CD. It has been suggested that TCR $\gamma\delta^+$ cells are spontaneously or highly reactive to mycobacterial antigens [27,28], although the data are not conclusive. For example, there have been conflicting reports of numbers of circulating TCR $\gamma\delta^+$ cells in patients with tuberculosis [37,38]. Furthermore, *in vitro* tests have shown that TCR $\gamma\delta^+$ cells do not respond equally to all mycobacterial preparations [30] and usually do not respond to PPD preparations (K. Pfeffer, personal communication). Indeed, one group has shown that TCR $\gamma\delta^+$ cells respond to live *Myco. tuberculosis* but not to identical organisms which have been killed and washed [39]. Furthermore, most demonstrations of TCR $\gamma\delta^+$ cell responses have been obtained with purified TCR $\gamma\delta^+$ cells. Such observations must raise some doubts as to the universality of TCR $\gamma\delta^+$ responses to mycobacterial antigens. We could find no evidence for mycobacterial responses by TCR $\gamma\delta^+$ T cells when cultured in presence of TCR $\alpha\beta^+$ cells in either control or patient groups. Thus, our data do not support the hypothesis that TCR $\gamma\delta^+$ are either 'first line' defence cells in anti-mycobacterial immunity or that TCR $\gamma\delta^+$ cell responses are altered in patients with CD.

In conclusion, our studies cast doubt on the mycobacterial theory of CD, and more specifically show no evidence to support the hypothesis that *Myco. paratuberculosis* plays a role in the pathogenesis of this condition. Although several groups have reported the detection of *Myco. paratuberculosis*

DNA in intestinal tissues of patients with CD by the PCR [11–14], other studies have failed to confirm these observations [15,16], even when using an extremely sensitive computerized technique [17]. Taken together, such observations increasingly undermine the hypothesis that CD has a mycobacterial etiopathology.

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