T cell responses to tuberculin purified protein derivative in primary biliary cirrhosis: evidence for defective T cell function

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Abstract

Background—Primary biliary cirrhosis (PBC) has an autoimmune aetiology, although little is known regarding the mechanisms of breakdown of self tolerance. One postulated mechanism of control of self tolerance is through interacting T cell subsets, a phenomenon explored in this study.

Aims—To characterise and compare T cell subset responses to an antigen (tuberculin purified protein derivative derived from mycobacteria) in PBC patients and controls. Cross reactive responses to mycobacteria have recently been implicated in the aetiology of PBC.

Subjects—58 PBC patients, 25 normal controls, and 34 chronic liver disease controls.

Methods—Responses to antigen were measured in terms of primary T cell proliferation and cytokine secretion (by ELISA). Responding cells were phenotyped by FACS analysis.

Results—Similar CD4⁺ T cell proliferative responses were seen in PBC patients (mean (SD) stimulation index (SI) 22.6 (27.2), 42 of 58 (72.4%) positive response), normal controls (46.5 (88.0), 17 of 25 (68% positive), and chronic liver disease controls (24.8 (49.8), 27 of 34 (79.4% positive)). Secretion of both interferon γ and IL10 was significantly lower in PBC patients than controls (IFN γ : PBC 822.7 (1100) pg/ml, controls 2929 (3402) pg/ml, $p < 0.05$; IL10: PBC 11.1 (15.6) pg/ml, controls 34.7 (63.4) pg/ml, $p < 0.05$).

Conclusions—In PBC unimpaired T cell proliferation is seen with reduced secretion of both Th-1 (interferon γ) and Th-2 type (IL10) cytokines. These findings may result from differential subset responses and may help explain the defects of functional immunity seen in PBC.

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Keywords: cirrhosis, autoimmunity, T lymphocyte, cytokine, tolerance, mycobacterial.

Primary biliary cirrhosis (PBC) is a chronic indolent condition characterised by immune mediated destruction of the biliary epithelial cells of the intrahepatic bile ducts. There is evidence to suggest that autoantibody and autoreactive T cell responses mounted against the members of the 2-oxo acid dehydrogenase

family of multienzyme complexes, in particular the E2 and protein X components of pyruvate dehydrogenase complex (PDC-E2/X), may play a part in the pathogenesis of PBC.¹ However, the mechanism by which self tolerance breaks down giving rise to this pathological autoreactive response is unclear.

One mechanism by which tolerance to self may normally be mediated is via interaction of the Th-1 and Th-2 subsets of CD4⁺ T cells, which exert reciprocal control through the secretion of cytokines.² Experimental disruption of these controlling interactions has been demonstrated to induce autoimmunity in animal models.³ Previous observations that PBC patients exhibit impairment of T cell 'suppressor' mechanisms,⁴ described before our current understanding of these important mechanisms of T cell mediated suppression was reached, raise the possibility that defective reciprocal control of CD4⁺ T cells may have a part to play in the breakdown of self tolerance in PBC. The experiments described here were designed to examine T cell responses to a physiologically relevant non-autoantigen in PBC, with particular attention being paid to the cytokine secretion patterns. Comparison of secretion patterns in PBC patients and controls allowed investigation of whether a shift in the normal Th-1/Th-2 response balance is seen in PBC, which might in turn suggest that imbalance in controlling subsets has a part to play in the pathogenesis of PBC.

The antigen chosen was tuberculin purified protein derivative (tuberculin PPD), an antigen preparation derived from mycobacterial cell walls. This antigen is of particular interest as it has recently been shown that serum samples from patients with PBC react with polypeptides of 70-65 and 55 kDa from *Mycobacterium gordonae*,⁵ leading to the suggestion that atypical mycobacterial infection may act, through the process of molecular mimicry, as a trigger for a breakdown of self tolerance in PBC. Despite the failure of a second study to confirm these findings⁶ the suggestion of a mycobacterial trigger for PBC has raised considerable interest. Neither of these studies, however, considered the question of T cell responses to mycobacterial antigens despite the evidence that T cell mediated mechanisms play an important part in the pathophysiology of PBC.¹ The secondary aim of the experiments described here was, therefore, to look for excess T cell responses to mycobacterial antigens in PBC suggestive of such cross reactivity.

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Methods

Patients

Peripheral blood T cell proliferative responses to mycobacterial antigens were measured in 58 patients with PBC (defined by the combination of cholestatic liver blood tests, positive histological diagnosis, and the presence of antimitochondrial antibodies to a titre $>1:40$ by immunofluorescence and anti-PDC-E2/X antibodies on enzyme linked immunosorbent assay (ELISA) testing as described previously).⁷ The mean (SD) age of the PBC patients was 61.6 (12.2) years (54 female). The control group consisted of 25 normal subjects (24 female, mean (SD) age 63.6 (12.2) years) and 34 patients with other forms of chronic liver disease (16 alcoholic liver disease, three primary sclerosing cholangitis, and 15 autoimmune hepatitis, 30 female, mean (SD) age 63.1 (12.6) years). Patients receiving immunomodulatory drug regimens were excluded from this study. None of the subjects studied had a history of clinical tuberculosis. Cytokine secretion patterns in response to PPD were measured in a further representative group of 17 PBC patients, 10 normal controls, and 11 chronic liver disease patients. All subjects gave informed consent.

Antigen

We used purified protein derivative (PPD) of *Mycobacterium tuberculosis* (tuberculin PPD 100 000 units/ml, Evans, Horsham, UK) in these experiments as it has been shown that there is biochemical similarity and immunological cross reactivity between PPDs derived from *M. tuberculosis* and the atypical mycobacteria.⁸ T cell proliferative and cytokine responses were measured to tuberculin PPD at a range of concentrations. Preliminary experiments suggested that peak responses were seen in the range of 1/200–1/2000 final dilution. All antigen preparations were made up in tissue culture medium (see later).

Tissue culture

All responses measured were those of peripheral blood T cells present in the mononuclear cell (PBMC) fraction. Blood (20 ml) was obtained aseptically from each subject by venepuncture and added to heparinised tubes (200 units of preservative free heparin (Sigma, St Louis, MO)). A further 10 ml of blood was used to prepare autologous serum for culture use. The mononuclear cell fraction was separated by standard Lymphoprep density centrifugation (Nycomed, Oslo, Norway) using Leucosep tubes (Greiner, the Netherlands). PBMC were washed three times in medium and cultured in 96 well U bottom plates (Costar, Cambridge, MA) at a density of 2×10^5 cells per well in 200 μ l of culture medium consisting of RPMI 1640 (Northumbria Biologicals, Cramlington, UK) supplemented with L-glutamine (Northumbria Biologicals, 2 mM final concentration) and 5% autologous serum prepared freshly and heat inactivated at 56°C for one hour. Repeat

experiments were performed in a sample of 14 PBC patients using pooled human AB serum (Sigma, St Louis, MO) in place of autologous serum to exclude significant serum effects. Triplicates of wells were prepared for each subject and antigen concentration. For each subject three control wells were set up containing cells but no antigen. Cells and antigen were co-cultured for six days at 37°C under 5% CO₂ in a humidified incubator.

Proliferation assay

Antigen responses were measured by proliferation assay at six days. Thirty seven KBq of [³H] thymidine (Amersham, UK) was added to each well in 30 μ l of culture medium. After a further 16 hours of culture the plates were filter harvested semi-automatically, dried for one hour, and counted on a Canberra-Packard Matrix 96 counter. Results are expressed as mean incorporated counts per minute (cpm) and stimulation indices (the stimulation index being the ratio of mean cpm in the antigen containing wells to mean cpm in control wells). A stimulation index of greater than 2.95 (mean control cpm +2SD) was taken as indicating positive response to PPD.

Phenotypic characterisation

Aliquots of proliferating T cells were characterised by immunofluorescence labelling and flow cytometry using a FACScan flow cytometer (Becton Dickinson immunocytometry systems, Oxford, UK). A single round of T cell restimulation and expansion in IL2 was used, for this experiment alone, to generate sufficient cell numbers for FACS analysis. Briefly, proliferating cells in a series of parallel primary culture to those outlined above were separated by density centrifugation (Lymphoprep, Nycomed, Oslo, Norway) on the third day in culture. Cells for FACS analysis were then cultured in 96 well U bottomed wells in culture medium containing 50 U/ml recombinant IL2 for a further seven days. At 10 days T cells were restimulated in 96 well U bottomed plates using irradiated autologous PBMC (in a 4:1 excess to T cells) and PPD at the same concentration as in the primary culture. On the third day proliferating cells were separated by density centrifugation and used for FACS analysis. Two colour flow cytometry analysis was performed as described previously⁹ using the lysis II software (Becton Dickinson). The cells were stained with optimum concentrations of the following monoclonal antibodies: anti-CD45 FITC/anti-CD14 PE (2D1/MoP9 Simultest Leucogate, Becton Dickinson), anti-CD3 PE, anti-CD4 PerCP, and anti-CD8 PE (all Becton Dickinson); 10^4 cells were counted per sample. The flow cytometer acquisition parameters were set up according to the manufacturers recommendations.

Cytokine assay

In separate experiments cytokine secretion in response to PPD was measured. PBMC

cultures were set up in triplicate as for the proliferation assay described above, and incubated at 37°C for four days. Culture supernatants from individual experiments were then pooled and stored at -80°C. All culture supernatants were subsequently thawed and assayed for IL2, IL4, IL10, and interferon γ by the use of sandwich ELISAs established and validated in our laboratory using standards from specific antibody suppliers. IL2 and IL4 were determined using the DuoSet antibody pairs (Genzyme Diagnostics, Cambridge, MA). IL10 determination utilised an antibody pair obtained from Pharmingen (San Diego, CA), namely monoclonal rat antihuman IL10 (clone JES3-9D7) and biotinylated rat antihuman IL10 (clone JES3-12G8). Interferon γ measurement used monoclonal mouse antihuman interferon γ and polyclonal rabbit antihuman interferon γ (Genzyme). Monoclonal capture antibody (50 μ l/well) was coated onto 96 well Immulon 4 ELISA plates (Dynatech Laboratories) (IL2 2.5 μ g/ml, IL4 2.0 μ g/ml, IL10 2.0 μ g/ml, and interferon γ 0.5 μ g/ml). After a blocking step, standards and samples (100 μ l/well) were incubated overnight at 4°C. Secondary detecting antibodies (100 μ l/well) were incubated for two hours at room temperature (biotinylated anti-IL2, IL4, IL10 at 1:25, 1:0, and 1:0 μ g/ml respectively with polyclonal rabbit anti-interferon γ at 1:500). These secondary antibodies (IL2, IL4, and IL10) were then detected with streptavidin-horseradish peroxi-

dase conjugate (Genzyme) (1:800) or, for interferon γ antirabbit IgG peroxidase conjugate (Sigma, Poole, Dorset, UK) (1:2000) (100 μ l/well) for one hour at room temperature, followed by colour development using the substrate *o*-phenyldiamine. Colour development was stopped with 2 M H₂SO₄ and the plates read at 492 nm.

Statistical analysis

Proliferative responses to tuberculin PPD are expressed as both mean incorporated counts per minute (cpm) in response to antigen and in control (antigen free) wells, and stimulation indices calculated from the ratio of the mean radioactivity incorporation in the antigen containing wells to that in the control wells. All cytokine concentrations are expressed in pg/ml. SI differences in response to tuberculin PPD and cytokine concentrations were all compared using a Mann-Whitney non-parametric *t* test. Frequencies of positive T cell proliferative response to the individual groups were compared using a Fisher's exact test.

Results

Proliferative responses

Background (control) incorporated counts per minute were not statistically different between the three subject groups (PBC 586 (570), normals 589 (577), chronic liver disease patients (CLD) 566 (606). Mean incorporated counts per minute in response to tuberculin PPD (data given for the tuberculin PPD concentration giving rise to the peak response) were PBC 7066 (8350), normal 9937 (13111), CLD 6626 (7243). The differences between the three subject groups were not statistically significant. Figure 1 shows the stimulation indices (SI) in response to tuberculin PPD for all three subject groups. Mean of the individual subject SIs was 22.6 (27.2) for the PBC patient group, 46.5 (88) for the normals, and 24.8 (49.8) for the CLD patients. The differences between the three groups were not statistically significant. Positive responses to tuberculin PPD were seen in 42 of 58 (72.4%) PBC patients, 17 of 25 (68.0%) normals, and 27 of 34 (79.4%) CLD. These differences were not statistically significant. No differences were seen between the different subject groups with regard to the concentration of tuberculin PPD giving rise to the peak proliferative response. Within the PBC patient group mean SI for patients with pre-cirrhotic disease was 23.7 (29.8), with 28 of 39 positive), while the mean SI for patients with cirrhosis was 20.2 (21.4), with 14 of 19 positive (Fig 2). None of the differences between cirrhotic and pre-cirrhotic patients were statistically significant. FACS analysis confirmed that the responding cells were CD4+, CD8-, CD3+ T cells (Fig 3). No phenotypic differences were seen in responding cells between PBC patients and controls. In all groups the proportion of non-T cells in the proliferating cell population was <5%, and the proportion of CD8+, CD4- cells in the T cell population was also <5%.

Figure 1: T cell proliferative responses to tuberculin PPD in PBC patients (PBC), normal controls (NORM), and patients with other chronic liver diseases (CLD) expressed as stimulation indices (SI). The stimulation index is defined as the ratio of ³H thymidine incorporation in antigen containing wells to that in control wells. Positive response is defined as an SI of greater than 2.95 (solid line).

Figure 2: T cell proliferative responses to tuberculin PPD in PBC patients with pre-cirrhotic (PRE) and cirrhotic (CIRR) disease. Differences between the two groups are not statistically significant.

Cytokine secretion

Culture supernatants pooled and stored at -80°C were thawed and assayed for the concentrations of IL2 and interferon γ (representative of Th-1 type cytokines) and IL4 and IL10 (representative of Th-2 type cytokines). IL2 was not detected in any of the control or PBC supernatant assayed. It is probable that the four day culture period, selected for the assay particularly of interferon γ and IL10, is not optimal for detection of IL2, which is both absorbed onto the surface of T cells and consumed in culture. Separate time course experiments designed to study the secretion of IL2 in culture in response to tuberculin PPD suggest a much earlier peak of IL2 secretion (at 24–36 hours) with no measureable IL2 in the culture supernatant beyond 48 hours (data not

shown). In contrast, interferon γ was found in high concentrations in the culture supernatants from most subjects (Fig 4). Mean interferon γ concentration in the PBC patient group was 822.7 (1100.5) pg/ml, which was significantly lower than that seen in the combined control group 2929 (3402.9) pg/ml, $p < 0.05$. No significant differences were seen in the control group between the CLD patients (3208.9 (3620.8) pg/ml) and the normal controls (2621.6 (3311.8) pg/ml, $p = \text{NS}$).

IL10 secretion in response to tuberculin PPD was considerably lower than interferon γ in all subject groups. Once again, however, secretion was significantly lower in the PBC patients (11.1 (15.6) pg/ml) than in the combined control group (34.7 (63.4) pg/ml, $p < 0.05$). No significant differences were seen again between the CLD controls (42.7 (87.0) pg/ml) and the normal subjects (25.8 (19.4) pg/ml, $p = \text{NS}$) (Fig 5). IL4 was undetectable in the culture supernatant in response to tuberculin PPD in all subject groups.

When pooled human serum was used in place of autologous serum as a culture medium co-factor in a sample of 14 PBC patients similar impairment of cytokine secretion was demonstrated (data not shown). These findings exclude the possibility that inhibitory factors in the autologous serum used in the culture medium are responsible for the impaired cytokine secretion.

Discussion

Many theories have been put forward to explain the mechanism by which the normal state of tolerance to self antigen breaks down giving rise to autoimmunity. One hypothesis is that autoreactive T cells are retained in the normal immune repertoire, but are subject to peripheral control mechanisms that prevent aberrant expression of pathological autoimmunity. Retention of this autoreactive potential may have the advantage that its controlled release in the appropriate context could allow self limiting cross reactive responses to be mounted, which can help eliminate pathogens expressing the cross reactive antigens.¹⁰ Pathological autoimmune responses would occur when the normal pathways of peripheral control break down. One such controlling mechanism seems to be the balance between the Th-1 and Th-2 subsets of CD4+ T cells, which support different effector responses and exert reciprocal control.² Th-1 cells secrete interferon γ and IL2 and promote macrophage activation. In contrast, Th-2 cells support mature antibody responses and secrete IL4 and IL10. Whereas in the mouse IL10 is exclusively secreted by Th-2 cells there is some evidence that in the human some limited Th-1 mediated secretion is seen.¹¹ Clarification of this issue is still awaited. The rat reconstitution experiments of Mason *et al*³ suggest that autoreactive CD4+ T cells are primarily of the Th-1 type, and are normally under the control of 'suppressive' Th-2 type cells. Previous experiments that have suggested that there is a generalised deficiency of T cell mediated suppression in

Figure 3: Sample FACSscan of responding cells from a PBC patient. FACS analysis shows that the cells proliferating in response to tuberculin PPD are predominantly CD4+, CD3+ T cells. Separate analysis shows cells to be CD8-. No phenotypic differences were seen in responding cells between PBC patients and controls.

Figure 4: Culture supernatant concentrations of interferon γ in PBC patients (PBC), normal controls (NORM), and chronic liver disease controls (CLD) on the fourth day in culture with tuberculin-PPD at peak concentration. Levels are expressed in pg/ml.

PBC¹² led us to postulate that a similar phenomenon of excess Th-1 activity, inadequately restrained as a result of defective Th-2 mediated suppression, may be one cause of the expression of T cell autoreactivity seen in PBC.¹ To examine the nature of the balance of Th-1 and Th-2 cell subset activity in PBC we studied responses to an antigen, tuberculin PPD, in terms of T cell proliferation and cytokine secretion. If our hypothesis, that immune responses in PBC are tipped towards the Th-1 response type, with the resultant expression of autoreactivity, through reduced Th-2 mediated control, were correct we would have expected to see increased IL2 and interferon γ secretion (Th-1) and decreased IL4 and IL10 secretion (Th-2). In practice we found decreased secretion of both Th-1 type (interferon γ) and Th-2 type (IL10) cytokines in PBC patients when compared with controls, suggesting that PBC is associated with a more complex impairment of CD4+ cytokine response.

Previous studies of cytokine function in PBC have looked at serum concentrations,^{13 14} spontaneous and mitogen induced secretion by mononuclear cells in culture,¹⁵⁻²² and secretion in the liver using in situ hybridisation for cytokine mRNA²³ and in vitro secretion by liver derived T cell clones.²⁴ Serum cytokine values are difficult to interpret, and are effected by clearance as well as synthesis rates. In addition, wide interindividual variation is commonly seen. Serum concentrations of IL2 and tumour necrosis factor have been shown to be increased in PBC patients when compared

with normal controls.¹⁴ A further study looking at IL1, IL6, tumour necrosis factor α , and interferon γ values showed similar increased values, but that the increase was common to all patients with CLD, possibly reflecting impaired excretion.¹³ The physiological relevance of cytokine secretion in response to mitogens is unclear. This notwithstanding, a pattern has emerged of reduced secretion of IL2 (in response to pokeweed mitogen¹⁵ and phytohaemagglutinin (PHA),²² although a further study has suggested a more complex impairment in the IL2 response),¹⁷ tumour necrosis factor (in response to endotoxin¹⁸ and PHA),¹⁹ and interferon γ (in response to PHA,^{19 20} and endotoxin).²¹ In the studies outlined here we have extended these investigations of cytokine secretion patterns from mitogen induced responses to those seen in response to a physiologically relevant recall antigen. The study of cytokine secretion in response to antigen in PBC has been limited to date. Gershwin and colleagues have shown that CD4+ PDC-E2 specific T cell clones derived from PBC patients are heterogenous in nature, some secreting IL2 and others IL4.²⁴ These findings suggest that there are both Th-1 and Th-2 CD4+ T cells present in the liver in PBC patients. PDC-E2 specific clones could not be derived from control livers and direct comparison between PBC patients and controls was not therefore possible. In situ hybridisation studies have demonstrated that mRNA for IL6, IL2, and interferon γ is present in the liver of both PBC patients and patients with autoimmune hepatitis.²³ mRNA for IL5, a Th-2 secreted cytokine was detectable in PBC patients but not AICAH controls.

There are two possible explanations for our finding of reduced secretion of both IL10 and interferon γ in PBC. Chronic liver damage occurring in PBC could theoretically, through a failure of hepatic clearance of agents toxic to T cells in culture, be giving rise to a state of toxic impairment of the T cell response. This seems unlikely for two reasons. Firstly, if CLD rather than PBC in itself is primarily to blame for the impairment in response then a similar pattern of impairment would be expected in the patients with comparable degrees of liver damage caused by non-autoimmune disease processes. This was not the case in our study. Secondly, the proliferative response of CD4+ T cells to tuberculin PPD (in contrast with the secretion of cytokines) is not impaired, suggesting that some aspects of the response to antigen are still normal. The combination of normal proliferation with impaired cytokine secretion suggests an alternative explanation for our findings. It is possible that there is a qualitative change in the nature of the immune response to tuberculin PPD occurring in PBC patients. The early phase of the response, with proliferation of CD4+ T cells to give healthy numbers of activated helper cells is unimpaired, but the maturation of the cells into functional activated cytokine secreting helper cells could be impaired. In this model PBC patients could thus be regarded as showing an 'immature' response to antigen that is

of CD4⁺ T cells to tuberculin-PPD is unimpaired in PBC, there seems to be a qualitative difference in the response, with impairment of secretion of both Th-1 and Th-2 type cytokines. This suggests that impairment of the normal effector aspect of the CD4⁺ T cell response may be occurring in PBC and may be responsible for some of the defects in functional immune responses seen in this condition.

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This work has been presented at the British Association for the Study of the Liver (London, 1995) and the American Association for the Study of Liver Disease (Chicago 1995), and published in abstract form. Jones DEJ, James OFW, Diamond AG, Bassendine MF. T-cell responses to mycobacterial extract (tuberculin-PPD) in patients with primary biliary cirrhosis. *Gut* 1995; **36**: A950. Jones DEJ, Palmer JM, Yeaman SJ, James OFW, Diamond AG, Bassendine MF. Cytokine responses to PPD in primary biliary cirrhosis: Evidence for a defect in CD4⁺ T-cell response. *Hepatology* 1995; **22**: 122A.

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