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T cell responses to natural human proteins in primary biliary cirrhosis

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SUMMARY

The study of T cell responses to autoantigens in human autoimmunity has been hampered by difficulties, firstly in identifying significant autoantigens, and secondly in the purification of authentic human proteins in sufficient quantities to allow characterization of antigen-specific T cell responses. In this study we have purified a human autoantigen, pyruvate dehydrogenase, retaining its enzymatic activity, and characterized autoreactive T cell responses to it in a human autoimmune disease, primary biliary cirrhosis. T cell responses to a mixture of the E2 and protein X subunits of human pyruvate dehydrogenase complex are seen in most affected patients, but in only a small minority of normal and chronic liver disease controls. By contrast, responses to whole pyruvate dehydrogenase complex occur with equal frequency in both groups. This suggests that responses to the E2 component/protein X of pyruvate dehydrogenase complex play a role in the pathogenesis of primary biliary cirrhosis. The availability of significant quantities of the human autoantigen in primary biliary cirrhosis makes this condition an interesting model in which to study true autoreactive human T cell responses.

Keywords liver cirrhosis biliary T cell autoimmunity autoreactive

INTRODUCTION

Although it is well established that human autoantibodies recognize the naturally produced, autologous antigens, similar proof for T cells has been harder to achieve. *In vitro* study of the immunological basis of many human autoimmune diseases has been hampered by failure to identify, and to isolate and purify, the relevant autoantigens in their natural form. Several different approaches have been used to compensate for the non-availability of natural human autoantigen in the study of autoreactive B and T cell responses, including the use of sequence-specific peptides [1–3], of recombinant proteins over-expressed in bacteria [4–6], and of equivalent proteins purified from tissue obtained from other species [7,8]. None of these approaches is wholly satisfactory, each having theoretical and practical disadvantages. In this study we have prepared and purified a natural human protein in sufficient quantity to allow us to characterize true autoreactive T cell responses in an important human autoimmune disease, primary biliary cirrhosis (PBC). This represents one of the first occasions in which T cell responses to a natural human autoantigen have been characterized in large numbers of patients with autoimmune disease, in comparison with suitable controls.

PBC is a chronic cholestatic liver disease with an autoimmune etiology [9]. The classic pathological lesion is of T cell-mediated destruction of the biliary epithelial cells of the small intrahepatic bile ducts [10]. Autoantibodies directed against a number of mitochondrial and nuclear antigens have been described in PBC [11,12]. The autoantibodies seen with the highest frequency in PBC are directed against antigens on the inner mitochondrial membrane, identified as the members of the 2-oxoacid dehydrogenase family of multi-enzyme complexes, in particular the dihydrolipoamide acetyltransferase (E2) component of pyruvate dehydrogenase complex (PDC) [13–16]. Previous studies looking at T cell responses to these antigens have been restricted to the use of bovine tissue-derived antigen [17–19], recombinant protein over-expressed in bacteria [19,20] and synthetic peptides [21]. Here we have purified natural human PDC from heart muscle, isolated the PDC-E1 and the co-purifying PDC-E2 and protein X components, and cleaved PDC-E2 into its constituent domains. Using these purified protein preparations we have, for the first time, studied and compared T cell responses to natural human autoantigens in PBC patients.

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SUBJECTS AND METHODS

Subjects

Peripheral blood T cell responses to PDC, PDC-E1 and PDC-E2/X were characterized in 28 female patients with histologically proven primary biliary cirrhosis. Mean age for the PBC patient group was 58·9 ± 9·7 years (range 39–76 years). The PBC group consisted of seven patients with stage IV disease (cirrhotic, mean age 63·8 ± 7·6 years, range 54–76 years) and 21 patients with stage I–III disease (pre-cirrhotic, mean age 57·3 ± 9·9 years, range 39–76

years). All 28 PBC patients were anti-E2 antibody-positive by ELISA (method described previously [22]). The control group consisted of 14 normal female subjects (mean age 58·8 ± 10·1 years, range 39–73 years), and 18 female patients with other forms of chronic liver disease (CLD) (12 with alcoholic liver disease, six with primary sclerosing cholangitis). Mean age of the CLD group was 60·0 ± 11·8 years (range 32–78 years). All control subjects were anti-E2 antibody-negative by ELISA. All subjects gave informed consent and local ethical approval was obtained. PBC patients and CLD controls on immunomodulatory therapeutic regimes were excluded from the study.

Cell preparation

Responses studied were those of T cells in the peripheral blood repertoire, allowing comparison between PBC patients and normal and CLD controls. Although the study of liver-infiltrating T cells might have been preferable, preliminary experiments suggested that it was not possible to obtain sufficient T cells from liver tissue to perform these experiments, even using explanted organs obtained at orthotopic liver transplantation**.** All subjects had adequate totals of peripheral blood lymphocytes. We have previously demonstrated that there are no significant differences in circulating T cell and $CD4^+$ subset counts between PBC patients and normal and CLD controls [23]. Blood (20 ml) was obtained aseptically from each subject by venepuncture and added to heparinized tubes (200 U of preservative-free heparin (Sigma, St Louis, MO)). A further 10 ml of blood were used to prepare autologous serum for culture use and for anti-E2/X antibody testing by ELISA. The mononuclear cell fraction was separated by standard Lymphoprep density centrifugation (Nycomed, Oslo, Norway). The mononuclear cells were washed three times in medium (as below) before culture.

Antigen preparation

PDC used as antigen in these experiments was extracted from human heart muscle obtained from explanted organs at cardiac allograft. The PDC-E1 and PDC-E2/X components were isolated from the whole complex by gel filtration. Human PDC was purified [24] using a modification of the method described by Stanley & Perham [25]. The final preparation was stored in 20 mM HEPES, 150 mM NaCl and 30% (v/v) glycerol, pH 7·4 (buffer A). This material revealed the five polypeptides characteristic of mammalian PDC when analysed by SDS–PAGE [26]. A fraction containing PDC-E2 and protein X was separated from E1 and E3 by resolution of the complex using gel filtration on a preparative Superose 6 (HR 16/50; Pharmacia, St Albans, UK) column at pH 9·0 in the presence of 1 M NaCl [27] and collected by ultracentrifugation at 250 000 ***g*** for 2 h at 2°C in a Beckman L5-65B ultracentrifuge. The pellet was resuspended and dialysed extensively against buffer A. PDC-E2 was split into its component catalytic and lipoyl domains by partial tryptic digestion as previously described [28]. Purities of all antigen preparations were analysed by SDS–PAGE, and protein concentrations were measured by the method of Bradford *et al.* [29]. Enzyme activities of both natural PDC and the reconstituted complex formed by the mixing of constituent subunits were measured by spectrophotometric assay of the reduction of NAD+ to NADH using the method described previously [30]. All protein preparations were stored at −20°C before use.

Culture

Peripheral blood mononuclear cells (PBMC) were cultured in 96-well U-bottomed plates (Costar, Cambridge, MA) at a density of 2×10^5 cells/well in 200 μl 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 1 h. Responses to PDC were measured at a range of concentrations, from 10 to 200 μg/ml, based on preliminary experiments which suggested that peak responses to PDC were in the concentration range 100–200 μg/ml. Responses to PDC-E1, PDC-E2/X and the catalytic and lipoyl domains of PDC-E2 were measured at a range of concentrations corresponding to the molarity used for PDC. Molarity calculations were based on published subunit molecular weights and stoichiometries [26]. The results given are for the peak response in each case. All antigen solutions were prepared in culture medium and filter sterilized before adding to culture wells. Filter sterilization of antigens in complete culture medium had no significant effect on antigen concentrations. Triplicates of wells were prepared for each subject and antigen combination. For each subject three control wells were set up containing cells but no antigen. Cells and antigen were co-cultured for 6 days at 37°C under 5% CO_2 in a humidified incubator.

Proliferation assay

Control and antigen responses were measured by proliferation assay at 6 days. ^{3}H-thymidine (37 kBq; Amersham, Aylesbury, UK) was added to each well in 30 μl of culture medium. After a further 16 h of culture, plates were filter harvested semi-automatically, dried for 1 h and counted on a Canberra-Packard Matrix 96 counter. Results are expressed as stimulation indices (SI), the SI being the ratio of mean ct/min in antigen-containing wells to mean ct/min in control wells.

Statistical analysis

Statistical analysis of SI was performed using the Mann–Whitney non-parametric test and Fisher's exact test. Correlations are by Spearman's rank correlation. An SI >2·85 (mean control ct/min + 2 s.d.) was taken as indicating a positive response to antigen.

RESULTS

Biochemical purification of human PDC, PDC-E1 and PDC-E2 gives rise to homogeneous preparations of human autoantigen [24]. PDC purified in this way retains enzymatic activity, assayed in terms of the reduction of NAD+ to NADH in the presence of its substrate pyruvate. Recombination of the fraction containing PDC-E2 and protein X with which it copurifies, with the −E1 and $-E3_1$ components derived using this purification process is followed by rapid re-formation of enzymatically active complex (data not shown).

T cell responses to human PDC-E2

Significant T cell proliferative responses to human PDC-E2/X were seen in most PBC patients, but in only a small minority of controls. Background proliferation measured in terms of mean counts incorporated per minute (ct/min) was not significantly different between the three subject groups (PBC patients $n = 28$, 363 ± 424, normal controls $n = 14$, 411 ± 457, CLD controls $n = 18$, 212 ± 214). Median SI in response to PDC-E2/X (data given for optimal response in terms of antigen concentration) were significantly higher in PBC patients than in the combined control

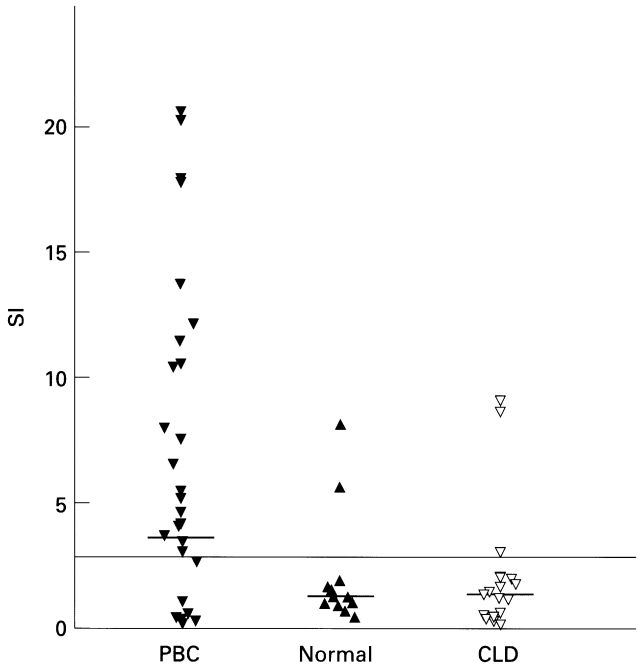

Fig. 1. Peripheral blood T cell responses to human pyruvate dehydrogenase complex (PDC)-E2/X in primary biliary cirrhosis patients (PBC), normal controls and patients with chronic liver disease (CLD). Values are for maximal stimulation index (SI) in each case**.** Median values for each subject group are indicated by a horizontal bar. Responses to human PDC-E2/X are significantly higher and more frequent in the PBC group than in control groups.

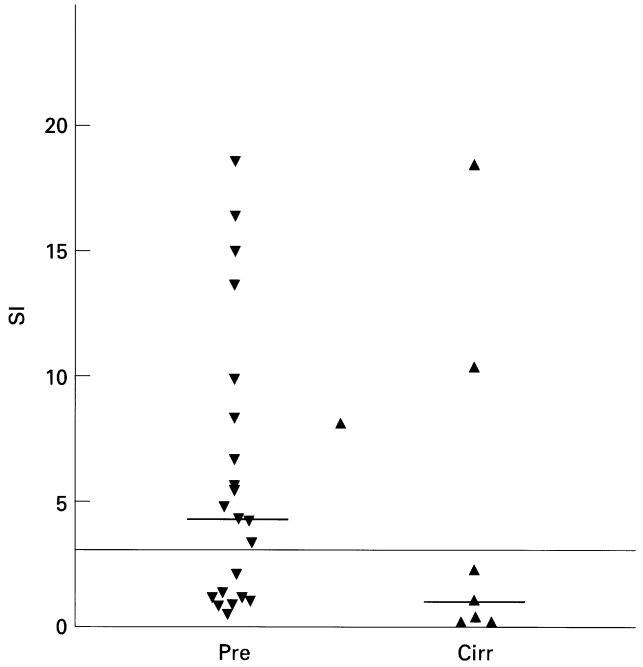

Fig. 2. Responses to human pyruvate dehydrogenase complex (PDC)-E2/X in primary biliary cirrhosis (PBC) patients with pre-cirrhotic (stages I–III) and cirrhotic (stage IV) disease. Values are for maximal stimulation index (SI) in each case. Median values for each subject group are indicated by a horizontal bar. Positive responses to E2/X are seen more frequently in pre-cirrhotic than cirrhotic patients, although this difference does not reach statistical significance.

group (PBC 3·7 (range 0·3–18·4), controls 1·3 (0·1–9·1), $P = 0·02$) (Fig. 1). The responses seen in the two control groups showed no significant differences between normals and CLD subjects (normals 1·3 (0·5–8·2), CLD 1·35 (0·1–9·1), $P = 0·9$). Of PBC patients, 15/28 (53·6%) had a positive response to human PDC-E2/X, compared with 5/32 (15·6%) controls. This difference was significant ($P = 0·003$). Within the control group two normal controls and three CLD controls showed positive responses to human PDC-E2/X. When PBC patients were subdivided according to disease stage, median SI in response to human PDC-E2/X was 4·2 (range 0·4–18·4) in patients with pre-cirrhotic PBC ($n = 21$), and 0·9 (0·3–18·1) in patients with cirrhosis ($n = 7$) (Fig. 2). Positive responses were seen in a higher proportion of patients with pre-cirrhotic disease than cirrhotic (13/21 *versus* 2/7), although this did not reach statistical significance.

T cell responses to human PDC

Unlike T cell proliferative responses to human PDC-E2/X, which were largely restricted to PBC patients, responses to whole human PDC were seen in a significant proportion of normal and CLD controls as well as the PBC population (Fig. 3). Median SI in response to PDC (data given again for peak response only) was 4·65 (range 0·4–30·0) in PBC patients and 3·5 (0·1–24·3) in the pooled controls ($P = 0·32$). Of PBC patients, 19/28 had a positive response to PDC compared with 16/32 controls ($P = 0·2$). No significant differences were seen in the control group between normal and CLD controls. The responses seen to the whole PDC may be accounted for by the responses seen to the isolated PDC-E1 component. Positive responses to PDC-E1 were seen in 20/28 PBC

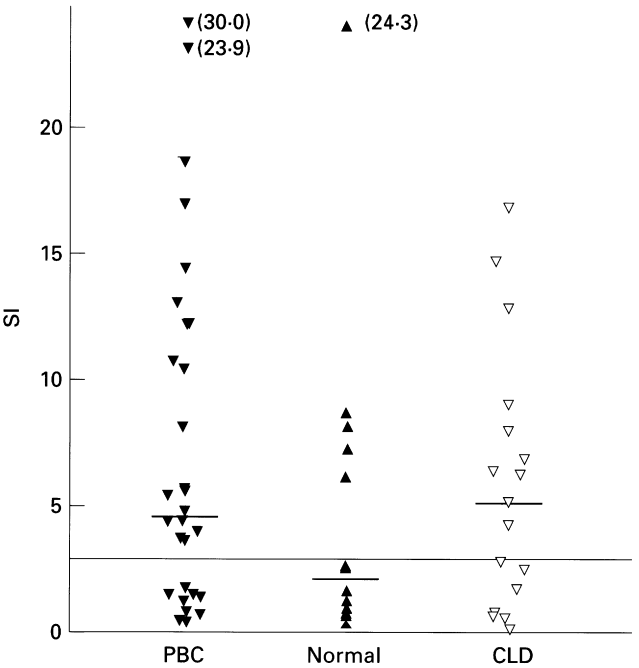

Fig. 3. Peripheral blood T cell responses of primary biliary cirrhosis (PBC) patients and controls to human pyruvate dehydrogenase complex (PDC). Values are for maximum stimulation index (SI) in each subject in response to PDC. Median values for each subject group are indicated by a horizontal bar. Differences in the frequency and magnitude of responses between groups are not significant. CLD, Chronic liver disease.

patients compared with 16/32 controls ($P = 0{\cdot}2$). Median SI were: PBC, 4·9 (range 0·2–20·7); normal controls, 2·2 (0·2–16·5); CLD controls, 3·5 (0·1–20·2).

Epitope localization within human PDC-E2

In order to localize significant T cell epitopes within PDC-E2, T cell responses were measured to the isolated domains of the protein. Cleavage and purification of the catalytic and lipoyl domains of PDC-E2 have been described previously [28], and highly purified preparations can be obtained using this biochemical approach (Fig. 4). Responses were measured using the same T cell proliferation assay approach in a further series of PBC patients ($n = 9$, three of whom had previously been demonstrated to be non-responders to human PDC-E2/X, and six to be responders) (Fig. 5). Positive responses were seen to PDC-E2 in 6/9 patients, matching the response pattern seen for these individuals in the initial PDC-E2 response experiments. In the human PDC-E2/X responding group, responses were seen to isolated lipoyl domain in 5/6 subjects and to isolated catalytic domain in 6/6 subjects. No responses were seen to either lipoyl or catalytic domain in the three subjects showing no responses to whole PDC-E2. These constitute an important internal negative control group.

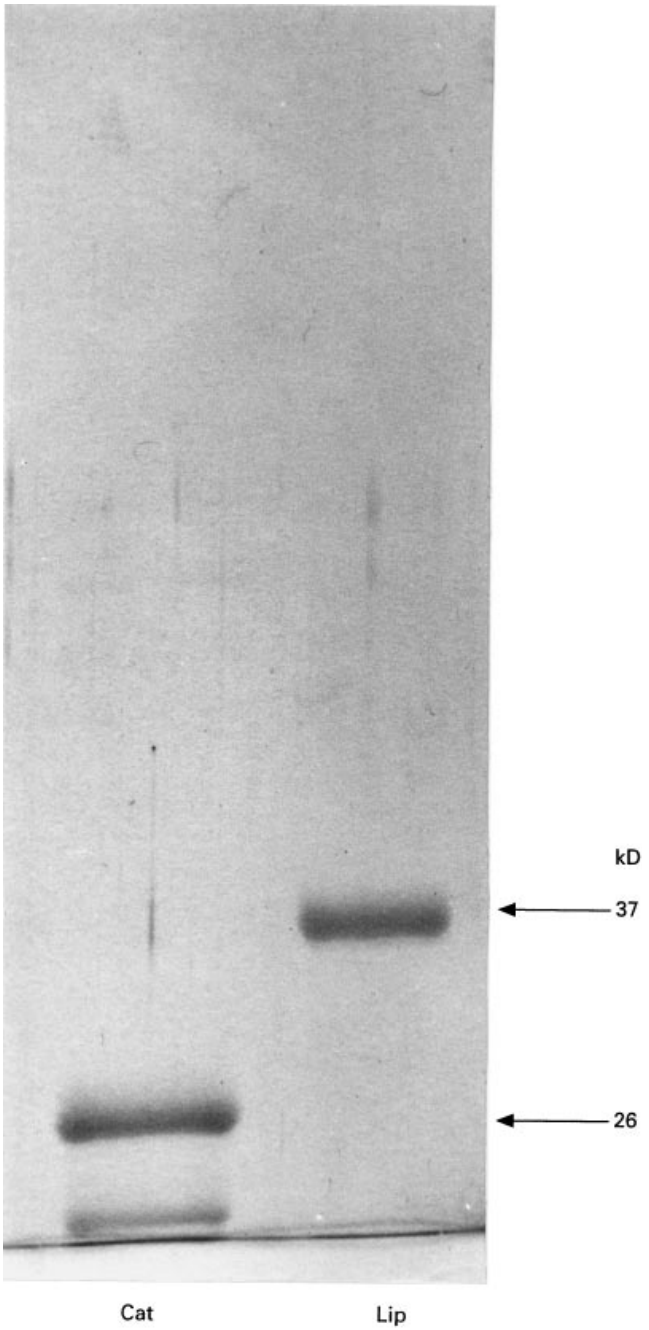

Fig. 4. Analysis of lipoyl and catalytic domains of human pyruvate dehydrogenase complex (PDC)-E2 on SDS–PAGE followed by staining with coomassie brilliant blue.

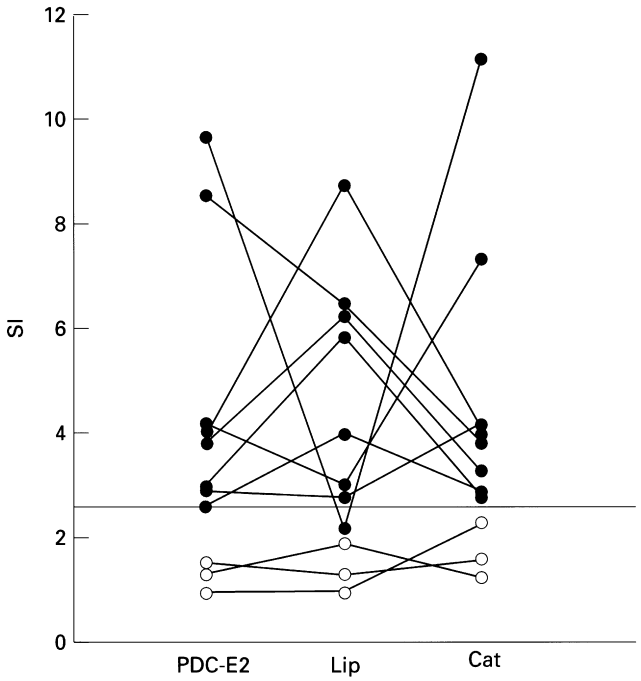

Fig. 5. T cell responses to human pyruvate dehydrogenase complex (PDC)-E2/X, isolated PDC-E2 lipoyl domain (Lip) and isolated human PDC-E2 catalytic domain (Cat) in a series of primary biliary cirrhosis (PBC) patients. Responses seen to each of the antigens in individual patients are linked by lines. Open symbols represent the responses seen to PDC-E2/X, lipoyl and catalytic domains in patients previously demonstrated to be non-responders to human PDC-E2/X. SI, Stimulation index.

DISCUSSION

In the experiments outlined here we have demonstrated autoreactive T cell responses to a tissue-derived, fully functional, human autoantigen in a human autoimmune disease, PBC. This represents one of the first demonstrations that T cell responses in human autoimmune disease are truly autoreactive, as responses have been measured to a natural, functional enzyme complex. Study of autoimmunity in humans has often been hampered by failure to identify the important autoantigens (e.g. in primary sclerosing cholangitis [31]), or by difficulties in obtaining purified autoantigen in sufficient quantity from human sources to make possible study of autoimmune mechanisms and epitope identification. Several approaches have been used to solve the problem of antigen availability. One approach, in the case of highly conserved antigens, is to use homologous antigen derived from an alternative

species. This approach has been used in the study of responses to acetylcholine receptor in myasthenia gravis, with electric organ acetylcholine receptor derived from the Torpedo substituting for the sparse and difficult to purify human skeletal muscle acetylcholine receptor [7,32], and indeed in earlier studies of PBC, which used bovine heart PDC as a substitute autoantigen [17–19]. For such studies to be relevant it is essential that there is adequate homology between the substitute antigen and its human equivalent. As the precise autoepitopes are unknown for most autoantigens, and single amino-acid variation within these epitopes can significantly alter peptide binding to MHC and presentation to the T cell receptor [33], the relevance of the response seen to even highly conserved foreign antigens to the actual autoreactive responses seen in the autoimmune state remains subject to doubt. The potential problems are highlighted by one study of T cell responsivity in myasthenia gravis, in which it was demonstrated that T cell lines raised to recombinant human acetylcholine receptor α-subunit failed to cross-react with antigen derived from Torpedo, the usual source of substitute antigen [32].

A second approach to antigen preparation is to use recombinant DNA technology to synthesize polypeptides in a foreign host once the relevant human gene has been cloned. Recombinant human protein has been used in the study of a number of autoimmune diseases, including PBC, in the studies of Gershwin and colleagues [19,20]. Although the use of recombinant protein has the advantage that there is sequence identity with human antigen, there are potential disadvantages. If the recombinant protein is overexpressed in bacterial systems then contamination with even minute quantities of bacterial protein or cell wall material can give rise to antigen preparations which can stimulate T cells and other mononuclear cells to proliferate, giving a spurious appearance of autoantigen specificity. Although eukaryotic expression systems help to solve this problem, the generation of large quantities of pure antigen often remains difficult. A second disadvantage of the recombinant approach is that the truncated nature of many of the proteins used, together with differences in folding and/or post-translational modification which occur in prokaryotic compared with eukaryotic systems, means that the tertiary structure of the recombinant antigen is likely to be different from that of the natural human protein. This may have important effects on entry into antigen-processing pathways and peptide derivation. Such alterations to processing, and hence presentation, may result in generation of 'silent' epitopes not normally presented and of limited physiological relevance. The use of peptides synthesized from the known sequence of an antigen gives rise to similar problems by bypassing the normal pathways of processing and presentation altogether.

In the experiments described here we have addressed these problems by studying T cell responses to a human autoantigen purified from human tissue. We have demonstrated that T cell responses to human PDC-E2/X, which are truly autoreactive in nature, are largely restricted to patients with PBC, being absent from all but a small minority of normal and CLD controls. It is important to note that the PDC-E2/X purified from human heart muscle in these experiments is intact, retaining enzymatic activity following *in vitro* reconstitution of the whole complex. The T cell responses detected in these experiments are therefore directed against autoepitopes processed by host antigen-presenting cells from a whole self-protein to which they are immunologically exposed *in vivo*. These findings complement and extend those from other studies using recombinant PDC-E2 [19,20], xenogeneic PDC-E2/X [17–19] and synthetic peptides [21], and confirm that the responses seen in these previous studies are indeed directed at the natural self-antigen and, taken together, these studies suggest that T cell responses to PDC-E2/X have an important role to play in the pathogenesis of PBC.

The human antigen preparation used in the experiments reported here contained both PDC-E2 and protein X (which co-purify). It was therefore not possible to determine whether T cell responses were directed against both PDC-E2 and protein X. The data regarding responses to recombinant PDC-E2 [19,20] and sequence-specific peptides [21], neither of which contained protein X-derived epitopes, suggest that at least some of the responses seen to PDC-E2/X are directed against the E2 component. There are some data available, however, to suggest that responses to protein X may be particularly important in biliary epithelial cell damage [34]. The true significance of T cell responses to protein X in PBC remains to be clarified.

We have extended the study of T cell responses to human PDC-E2 in PBC by attempting to localize the significant T cell epitopes within the molecule. T cell responses were measured to isolated 'lipoyl' (containing the N-terminal inner and outer lipoyl domains joined by a flexible linker sequence) and 'catalytic' (E1/E3 binding and catalytic domains similarly linked) domains of PDC-E2 generated from the whole molecule by partial tryptic digestion [28]. Responses were seen to both the lipoyl and the catalytic domains in a series of PBC patients showing positive responses to human PDC-E2/X, suggesting that there are T cell autoepitopes in both the lipoyl binding and catalytic regions of the polypeptide. The lack of response to either the lipoyl or the catalytic domain in the PDC-E2/X non-responsive PBC patients forms an important negative control, and confirms that cryptic epitopes are not generated by the partial tryptic digestion process. The wider distribution of T cell epitopes within PDC-E2 is in marked contrast to the B cell epitopes which are largely restricted to the inner lipoyl domain, and absent from the catalytic domain [35,36]. The observation that T cell autoepitopes are not limited to the lipoyl domains is in keeping with two previous studies, which have demonstrated, first, that some PBC patients responsive to bovine PDC-E2 show no T cell response to recombinant lipoyl domain [19], and second, that primary proliferative T cell responses are seen to a pool of synthetic peptides spanning the catalytic domain [21]. One possible explanation for the wide distribution of T cell autoepitopes in PDC-E2 is that it results from epitope spreading within the antigenic protein, a phenomenon demonstrated in other autoimmune responses, most notably in the responses to glutamic acid decarboxylase in the non-obese diabetic (NOD) mouse [37]. The patients studied here all had relatively advanced, and hence 'immunologically mature', disease. We are currently extending this study to patients in the earliest stages of the disease to see whether a more limited T cell epitope distribution occurs.

The PBC-specific nature of the T cell response to human PDC-E2/X is in contrast to the response to human PDC itself, which is seen in significant numbers of normal and CLD controls, as well as PBC patients. This response to the whole complex in all subjects is directed primarily against the E1 component. T cell responses to other autoantigens have been noted in apparently normal individuals [7,8,32,38–42], although previous studies in PBC are contradictory, with different studies showing responses to bovine PDC and PDC-E1 to be both present [17] and absent [18,20] in control subjects. Such observations in PBC and other autoimmune diseases suggest that thymic deletion of some potentially autoreactive T cells is incomplete, possibly resulting from low autoepitope

affinity for MHC class II leading to low-avidity T cell recognition and escape from clonal deletion [43]. The low incidence of pathological autoreactivity in the general population suggests, in turn, that effective mechanisms must exist for the peripheral control of these potentially harmful cells. It has been suggested that retention of these autoreactive cells, far from being coincidental, has a physiological role in providing a repertoire of cells which can cross-react with microbial homologues of the self-protein to which they are reactive. This has the effect of providing an 'instant immunological memory', allowing effective and rapid immunological response to microbial infection [44]. It is perhaps relevant that patients with acute tuberculosis appear to mount anti-PDC responses, with the inherent potential for autoreactivity, in response to mycobacterial infection [45].

In this study we have uniquely used natural human protein to identify significant T cell autoantigens in a human autoimmune disease. The identification and characterization of the autoantigens in PBC may allow the future use of immunotherapeutic approaches to the treatment of this difficult and unpleasant disease.

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