

Extensive Homology between the Major Immunodominant Mitochondrial Antigen in Primary Biliary Cirrhosis and *Helicobacter pylori* Does Not Lead to Immunological Cross-reactivity

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Background: Primary biliary cirrhosis (PBC) is an immune-mediated chronic cholestatic disease characterized by the presence of antibodies directed predominantly against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2). What provokes tolerance breakdown in PBC remains to be established, though there is evidence to indicate that microbes may induce anti-mitochondrial antibodies (AMA) through a mechanism of molecular mimicry. **Methods:** Having found that urease beta (UREB)_{22–36} antigen of *Helicobacter pylori* (HELPHY) shares extensive (87%) similarity with PDC-E2_{212–226}, the major mitochondrial autoepitope, it was hypothesized that this would also lead to cross-reactivity. The UREB/PDC-E2 mimics were thus constructed and tested by ELISA in 112 PBC patients and 114 controls. **Results:** Reactivity to PDC-E2_{212–226} was found in 104 patients but to UREB_{22–36} in only 2. In these two patients, the double reactivity was not cross-reactive. The lack of surface antibody accessibility to UREB_{22–36}, as demonstrated through three-dimensional model prediction analysis, may explain this unexpected finding. There was some speculation on whether HELPHY UREB_{22–36} might act as a cross-reactive CD4 T-cell epitope. All seven PBC patients, tested in a standard proliferation assay against PDC-E2_{212–226}, gave a positive response. All seven were unresponsive to HELPHY UREB_{22–36}. The pattern of reactivity to HELPHY antigens by immunoblot was similar between anti-PDC-E2-positive and negative PBC cases, as well as between PBC patients and controls. **Conclusion:** Contrary to common belief, extensive sequence homology (molecular mimicry) between self and microbe does not necessarily result in cross-reactivity. It is therefore likely that, when present, cross-reactivity between self and microbes is of biological importance.

Key words: Autoantibody; autoimmune disease; autoimmunity; B-cell; cholestasis; hepatitis; mimicry; tolerance; similarity; T cell

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Primary biliary cirrhosis (PBC) is an immune-mediated chronic cholestatic disease characterized by the presence of anti-mitochondrial antibodies (AMA) directed predominantly against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) (1, 2).

What provokes tolerance breakdown in PBC remains to be established (3), though there is evidence to indicate that microbes may induce PBC-specific autoimmunity through a mechanism of molecular mimicry (4–7). *Helicobacter pylori* (HELPHY) and *Helicobacter hepaticus* (HELHEP) have recently been implicated in the pathogenesis of PBC, since microbial DNA has been found in liver tissue and antibodies to the microbe in the serum of patients with PBC (8–13).

In a recent study by Amedei et al. (14) it was reported that

H. pylori-infected patients with gastric autoimmunity harbour in vivo-activated gastric CD4⁺ T cells that recognize the gastric H⁺K⁺-ATPase proton pump, the major autoantigen in autoimmune gastritis, and HELPHY antigens. The observation that T cells cross-reactively recognizing sequences in 9 HELPHY proteins and in mimics in the proton pump display the tissue-damaging T helper 1 profile has led to the proposal that infection with HELPHY activates cross-reactive T cells capable of inducing gastric autoimmunity through a mechanism of molecular mimicry (14).

In the present study we speculated on whether HELPHY antigens and PDC-E2 are targets of cross-reactive immunity in PBC (7). Our assumption found theoretical support when, through a search of the protein database, we found a strong

amino acid similarity between PDC-E2₂₁₂₋₂₂₆, the immunodominant B- (15, 16) and T-cell (17, 18) autoepitope on PDC-E2 and a short sequence on the urease beta (UREB) subunit, a protein that, along with urease alpha (UREA), represents a major focus of anti-HELPHY immunity (19–22). We therefore investigated whether the HELPHY UREB mimic and PDC-E2₂₁₂₋₂₂₆ are targets of cross-reactive response at the B- and CD4 T-cell level (5, 16, 17, 23, 24). In the search for disease-specific anti-microbial reactivity, we have also investigated humoral immune responses to HELPHY antigens in patients with PBC in comparison with those in a large number of controls including patients with viral hepatitides, autoimmune thyroiditis, lupus patients and healthy subjects.

Patients and Methods

Patient population

Serum samples were obtained from 112 patients with PBC (mean age 49.2 years, range 27–85; 99 F), all but 15 AMA positive by indirect immunofluorescence (IIFL), (median titre: 1/1280, range 1/40–1/10,480), mean duration of disease 67.2 months (range 1–227) attending the outpatient clinic of the Liver Transplantation and Hepatobiliary Medicine Unit, Royal Free Hospital, London. Demographic, biochemical and clinical characteristics of the patients are given in detail in previous reports (6, 16, 25–27). All sera were tested under code.

Pathological controls included 98 AMA-negative patients (54.8 years, range 24–85, 89 F) and all those positive for anti-HELPHY antibodies by ELISA and immunoblotting including 25 with chronic hepatitis B virus (HBV) infection, 31 with chronic hepatitis C virus (HCV) infection; 23 with autoimmune thyroid disease and 19 with systemic lupus erythematosus.

Sixteen healthy, AMA-negative volunteer members of staff (mean age 38.1 years, range 22–58, 13 F), all anti-HELPHY antibody positive, were tested as normal controls. AMA was detected by conventional IFL, immunoblotting using human liver mitochondrial fraction (Euroimmun, Pontypool, UK) and ELISA using purified PDC from porcine heart mitochondria (Euroimmun) as antigen.

Antibodies to HELPHY were tested by commercially available ELISA and immunoblotting using a purified HELPHY antigen extract containing UREB as the antigen, in accordance with the manufacturer's instructions (Euroimmun).

The project was approved by the local ethics committee.

Protein database search

The UREB HELPHY homologue of human PDC-E2₂₁₂₋₂₂₆ was identified by scanning the SwissProt protein database using the *ProteinInfo* program (6, 16). Amino acid comparison of full-length human PDC-E2 and HELPHY UREB was carried out using the *BLASTp* 2 sequences protein–protein comparison program (6, 16). The observed similarities were

analysed in relation to those between human PDC-E2 and UREA HELPHY, UREB HELHEP or UREA HELHEP.

Peptide synthesis

Three 15-mer peptides containing the relevant human inner PDC-E2₂₁₂₋₂₂₆, PDC-E2₉₁₋₁₀₅, and HELPHY UREB₂₂₋₃₆ mimics and an irrelevant 15 aa control peptide (-YVNQSLRPTPLEISV-) were constructed (Mimotopes Ltd., Clayton, Australia).

ELISA

Antibody binding to the peptides was determined by ELISA (6, 16, 28) using a serum sample dilution of 1/200. Reaction for a given peptide was considered positive when $OD^{test}/OD^{control}$ peptide was ≥ 2 (6, 16, 29).

Inhibition studies

Peptides. To investigate whether the simultaneous reactivity to HELPHY UREB₂₂₋₃₆ and human PDC-E2₂₁₂₋₂₂₆ was due to cross-reactivity, competition ELISAs were performed (6, 16, 28–30), measuring residual anti-HELPHY UREB₂₂₋₃₆ or anti-PDC-E2₂₁₂₋₂₂₆ reactivity after incubation, with HELPHY UREB₂₂₋₃₆, PDC-E2₂₁₂₋₂₂₆ and a control peptide as the liquid-phase competitors.

Antigens. The possibility that PDC-E2 and HELPHY UREB are targets of cross-reactive antibody responses at the level of full proteins has been addressed through inhibition studies investigating inhibition of antibody reactivity to human liver PDC-E2 (Euroimmun) on immunoblot using a purified HELPHY extract containing the UREB subunit as a solid-phase competitor; conversely, antibody recognition of UREB has been tested by immunoblot before and after solid-phase incubation of serum samples with PDC complex from porcine heart mitochondria (Euroimmun).

Three-dimensional modelling. Three-dimensional modelling of the relevant HELPHY UREB₂₂₋₃₆ was carried out by analysing the structure of UREA-UREB HELPHY complex (MMDB Code: 17609) with the Cn3D visualization tool.

Proliferation assays. Proliferative responses to the relevant HELPHY UREB₂₂₋₃₆ and PDC-E2₂₁₂₋₂₂₆ peptides, PDC antigen, have been investigated in 7 PBC patients, and 5 pathological controls (4 with HCV, 1 with alcoholic hepatitis), all female (median age 54, range 37–68), all positive for anti-HELPHY antibodies. Fresh peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient solution (Amersham Pharmacia Biotech UK, Little Chalfont, UK) centrifuged at 800g for 20 min at 20 °C. The layer of PBMC was removed to a fresh tube and washed twice at 600g for 10 min with RPMI1640 medium (Autogen Bioclear UK Ltd., Calne, UK).

The medium used for all cell cultures was AIM-V (Invitrogen, Paisley, Scotland, UK) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/mL benzyl penicillin and 0.1 mg/mL streptomycin, 2.5 µg/mL amphotericin

Antigen	aa	Sequence	Similarity (%)	Identities
Human PDC-E2 (ILD)	(212-226)	K L S E G D L L A E I E T D K	13/15 (87%)	7
HELPHY UREB	(22-36)	<i>R L G D T D L I A E V E H D Y</i>		
HELPHY UREB	(463-477)	A N A S I P T P Q P V Y Y R E	7/15 (47%)	6
Human PDC-E2	(293-307)	<i>V A A V P P T P Q P L A P T P</i>		
HELPHY UREB	(463-477)	A N A S I P T P Q P V Y Y R E	8/15 (53%)	5
Human PDC-E2	(283-297)	<i>P Q V P P P T P P P V A A V P</i>		
HELPHY UREA	(1-14)	M K L T P K E L D K L M L H Y	9/15 (60%)	5
Human PDC-E2	(424-438)	<i>V L L V R K E L N K I L E G R</i>		
HELHEP UREB	(20-34)	R L G D T N L F A E I E K D Y	13/15 (87%)	6
Human PDC-E2 (ILD)	(212-226)	K L S E G D L L A E I E T D K		

Fig. 1. Sequence alignment between human E2 subunit of human pyruvate dehydrogenase complex (PDC-E2), and urease B (UREB) and urease A (UREA) of *Helicobacter pylori* (HELPHY) and *Helicobacter hepaticus* (HELHEP). Amino acids in standard single-letter code. Identities in **bold**; Conservative substitutions in *italics*; ILD = inner lipoyl domain.

B (Fungizone, Invitrogen) with 5% heat inactivated foetal calf serum (FCS, Sigma-Aldrich, Dorset, UK).

Fresh PBMC, 1×10^6 /mL, was diluted in a complete culture medium and 200 µL/well was added in a 96-well round-bottomed plate along with 10 µMol individual peptide or 5 µMol PDC antigen (Sigma-Aldrich) or non-antigen-specific stimuli (2 µg/mL PHA) served as positive control. After a 7-day culture at 37°C in a humidified 5% CO₂ atmosphere, the microcultures were pulsed with 0.5 µCi/well of ³H-thymidine for 18 h and harvested onto glass-fibre filter papers using a multichannel harvester. In each experiment, 5 wells containing PBMC, but no antigen served as negative controls. Each peptide or full-length antigen was tested in triplicate. The amount of radioactivity incorporated into synthesized DNA was measured in a MicroBeta counter (EG&G Wallac, PerkinElmer Life Sciences UK Ltd., Cambridge, UK). Results were expressed as the stimulation index (SI), which is the ratio of the mean counts per minute (cpm) from triplicate determinations with antigen to the mean cpm obtained without antigen. The wells exhibiting a mean SI >2.5 were considered positive (17).

Statistical analysis

Results are presented as means ± s_x (standard error of the mean) or as percentages (%). Comparison between values was done using the chi-squared test and the Fisher exact test, as appropriate. A two-tailed P value ≤0.05 was considered significant. Correlations between variables were assessed

using the Spearman rank order correlation coefficient. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, Ill., USA) statistical package.

Results

Protein database search

BLASTp 2 sequence comparison revealed the best similarity between the 569 aa long UREB protein and the inner lipoyl domain of PDC-E2 to involve the immunodominant PDC-E2₂₁₂₋₂₂₆ epitope. HELPHY UREB₂₂₋₃₆ shares 13/15 (87%) similarity (7 identities) with the human PDC-E2₂₁₂₋₂₂₆ (Fig. 1).

Two other HELPHY UREB sequences with similarity to PDC-E2 involved regions with a lesser extent of similarity, 47% (6 identities) and 53% (5 identities) that did not represent PDC-E2 epitopes (Fig. 1).

The best homology shared by UREA HELPHY and PDC-E2 was an 8/15 (53%) similarity (5 identities) between HELPHY UREA₁₋₁₄ and PDC-E2₄₂₄₋₄₃₈ (Fig. 1); no significant similarity existed between the UREA HELPHY and the PDC-E2₂₁₂₋₂₂₆.

The corresponding sequence on UREB of *H. hepaticus* also shared homology with human PDC-E2₂₁₂₋₂₂₆, though to a lesser extent compared with that of HELPHY (Fig. 1).

Reactivity to peptides and antigens

Reactivity to human PDC-E2₂₁₂₋₂₂₆ was found in a total of

104 PBC patients: 94 (97%) of 97 PBC cases, positive for AMA by IFL, and 10 (67%) of 15 PBC cases, negative for AMA by IFL.

Among the 104 anti- PDC-E2₂₁₂₋₂₂₆-positive patients, reactivity to HELPY UREB₂₂₋₃₆ was found in 2 (2%) and reactivity to the HELPY UREB full-length protein in 72 (69%) including the 2 anti-HELPHY UREB₂₂₋₃₆ positive cases. Among the 10 anti-PDC-E2₂₁₂₋₂₂₆ negative patients, there was no reactivity to HELPY UREB₂₂₋₃₆, 7 (70%) reacting with the full HELPY UREB full-length protein.

Among the 98 pathological controls, reactivity to human PDC-E2₂₁₂₋₂₂₆ was found in 3 (3%) including 2 patients with hepatitis C and one with systemic lupus erythematosus (SLE); and to HELPY UREB₂₂₋₃₆ in 2 (one with HBV and one with HCV) not reacting to human PDC-E2₂₁₂₋₂₂₆. There was no correlation of antibody reactivity to PDC-E2₂₁₂₋₂₂₆ or full PDC-E2 and individual HELPHY antigens (Fig. 2A) including UREB or UREA. The pattern of reactivity to HELPHY antigens by immunoblot was similar between anti-PDC-E2 positive and negative PBC cases, as well as between PBC and controls (Fig. 2B).

Inhibition studies

Peptides. Inhibition of reactivity to human PDC-E2₂₁₂₋₂₂₆/

HELPHY UREB₂₂₋₃₆ mimics by pre-incubation with the relevant peptides of the serum samples from the two microbial/self double-reactive PBC cases is detailed in Fig. 2. Antibody binding to HELPHY UREB₂₂₋₃₆ was inhibited in up to 89% of cases after incubation with the HELPHY UREB₂₂₋₃₆ peptide but not PDC-E2₂₁₂₋₂₂₆ or the control peptide (Fig. 3A, B).

Antigens. Incubation of a PDC-E2₂₁₂₋₂₂₆/HELPHY UREB₂₂₋₃₆ double-reactive serum sample with UREB HELPHY antigen as a solid-phase competitor was able to abolish reactivity to human UREB HELPHY but did not have an effect on reactivity to PDC-E2 (Fig. 3C). Conversely, incubation of double-reactive sera with PDC antigen inhibited reactivity to PDC-E2 but did not diminish reactivity to HELPHY UREB (Fig. 3D).

Three-dimensional modelling. Three-dimensional modelling predicts the core of HELPHY UREB₂₂₋₃₆ not to be exposed on the surface of the UREA/UREB complex (Fig. 4).

Proliferation assays. Proliferative responses to peptides and antigens are summarized in Fig. 5. Stimulation of PBMC with the non-specific stimulator PHA resulted in strong proliferative responses in PBC patients (mean SI ± s_x: 22.3 ± 4.64) and pathological controls (13.6 ± 2.25).

Proliferative responses to the PDC-E2₂₁₂₋₂₂₆ peptide were

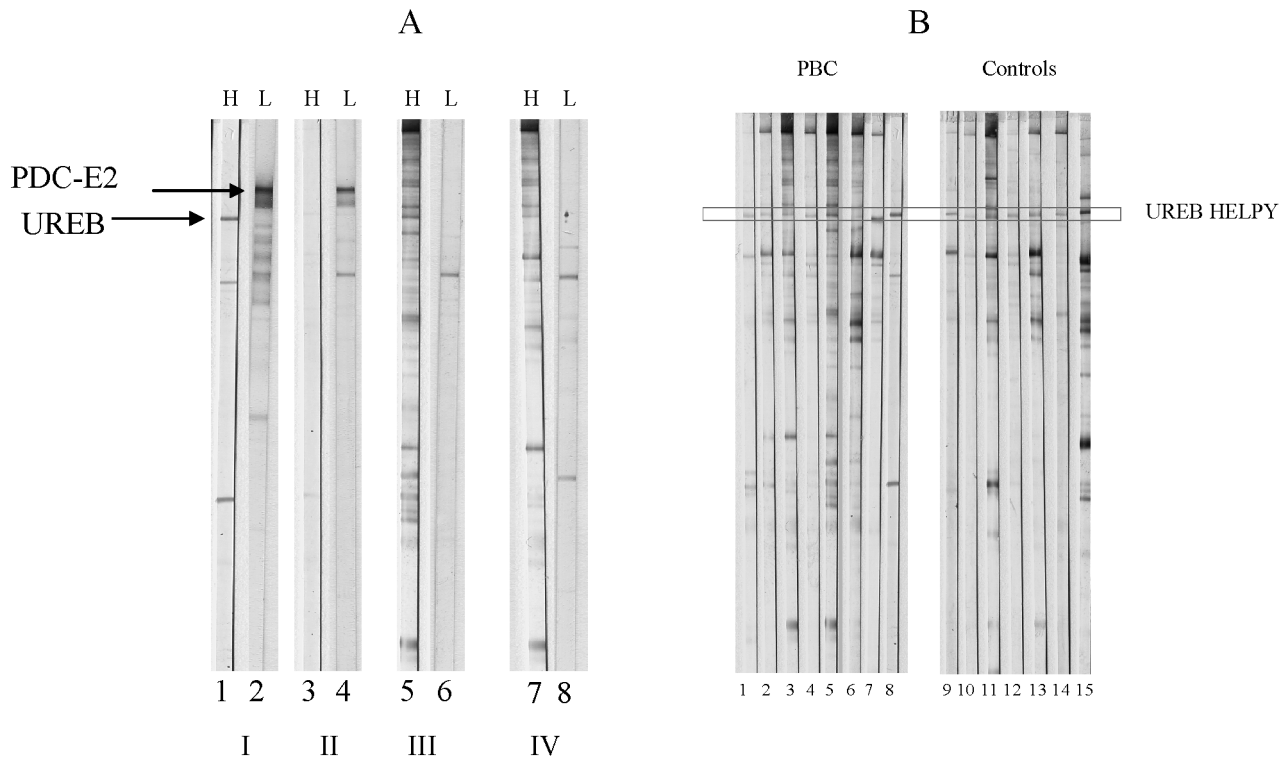


Fig. 2. Patterns of reactivity to *Helicobacter pylori* (HELPHY) and pyruvate dehydrogenase complex (PDC) antigens. A. Antibody reactivity to HELPHY (H) and human liver (L) PDC antigens in representative PBC cases. Notice the presence of reactivity to both urease beta (UREB) (lane 1) and PDC-E2 (lane 2) in the serum sample (I); absence of reactivity to UREB (lane 3) in the presence of reactivity to PDC-E2 (lane 4) in another PBC case (II); and presence of reactivity to UREB (lanes 5 and 7) in the absence of reactivity to PDC-E2 (lanes 6 and 8) in two other PBC cases (III and IV). B. Patterns of reactivity to HELPHY antigens in PBC cases—PDC-E2 positive (lanes 1–4) or negative (5–8) and controls (9–15).

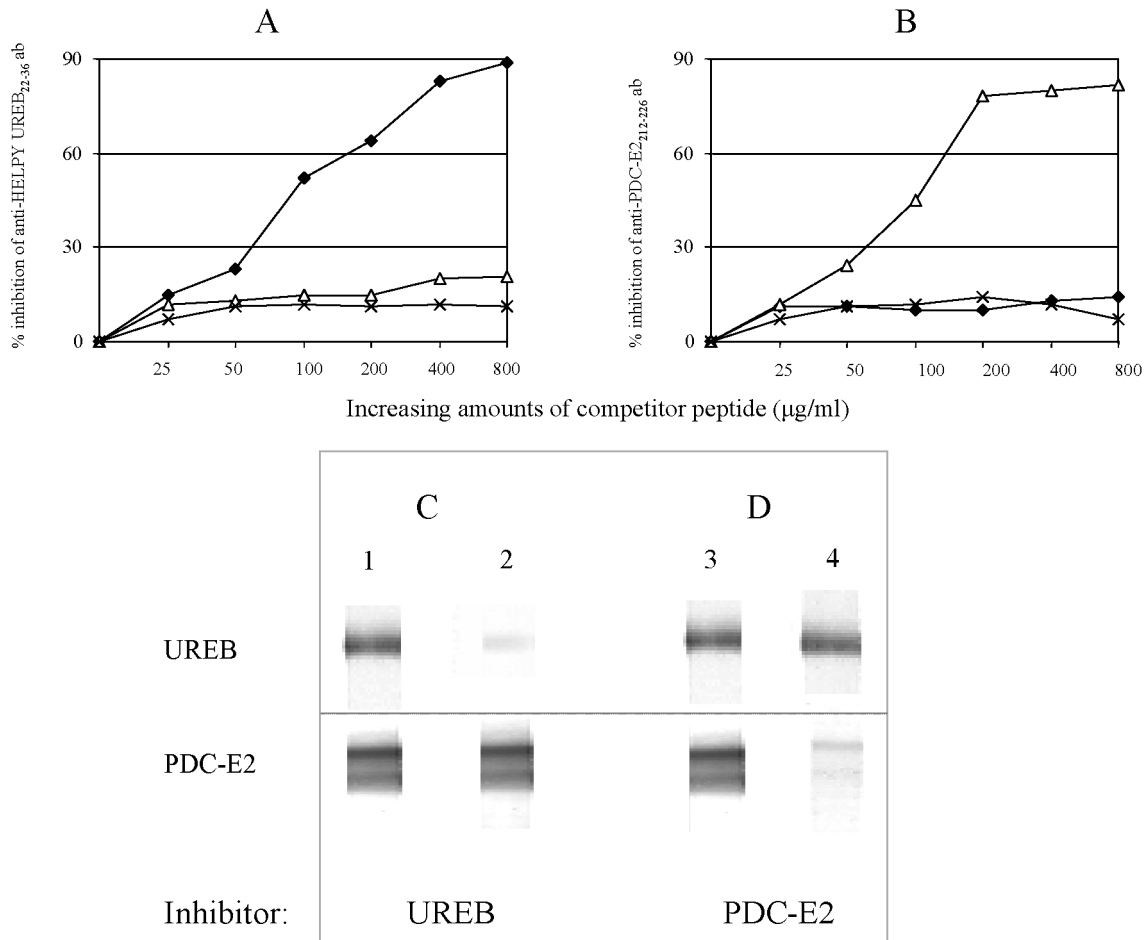


Fig. 3. A–B. Inhibition of antibody binding against HELPHY UREB₂₂₋₃₆ (A) and human PDC-E2₂₁₂₋₂₂₆ (B). Pre-incubation with HELPHY UREB₂₂₋₃₆ (◆), human PDC-E2₂₁₂₋₂₂₆ (Δ), and control peptide (×) of a HELPHY UREB₂₂₋₃₆/PDC-E2₂₁₂₋₂₂₆ double-reactive serum sample. On the vertical axis is the percentage of inhibition of antibody binding to the peptide in the presence of competitor peptide or antigen at different concentrations: 0; 25; 50; 100; 200; 400; 800 µg/mL. C–D. Antibody binding against HELPHY UREB (top band) and human PDC-E2 (bottom band) before (lanes 1 and 3) and after pre-incubation (2 and 4) with the HELPHY extract antigen (C) or PDC antigen (D) of a HELPHY UREB/PDC-E2 double-reactive serum. Notice that HELPHY antigen as inhibitor is able to abolish reactivity to human UREB HELPHY (lane 2, top) but does not have an effect on reactivity to PDC-E2 (lane 2, bottom). Conversely, incubation of double-reactive sera with PDC antigen inhibits reactivity to PDC-E2 (lane 4, bottom) but does not diminish reactivity to HELPHY UREB (lane 4, top). HELPHY = *Helicobacter pylori*; PDC = pyruvate dehydrogenase complex; UREB = urease beta.

found in 6 of the 7 PBC patients (3.03 ± 0.1) and 1 (2.6 ± 0.23) of 5 pathological controls (1.66 ± 0.17)—a 53-year-old woman with chronic HCV infection negative for AMA. All seven of the PBC patients (7.67 ± 2.8) and one control (the 53-year-old HCV-infected woman) also recognized the PDC antigen. No reactivity was found to the HELPHY UREB₂₂₋₃₆ in PBC patients (1.44 ± 0.07) or pathological controls (1.24 ± 0.07) (Fig. 5).

Discussion

In the present study we have investigated the role of molecular mimicry (5, 24, 31) between *H. pylori* and PDC-E2, the major mitochondrial autoantigen in PBC and found no evidence of cross-reactive immunity in PBC patients.

This finding was surprising considering the degree and the nature of the microbial/self-mimicry, as well as the experimental evidence linking HELPHY infection to PBC. Not only do HELPHY and PDC-E2 share a striking similarity in molecular mimicry terms (5), but also the microbial sequence originates from UREB, an antigen which, in complex with UREA, is a key target of anti-HELPHY immunity (20–22, 32). Moreover, the best similarity between microbe and self involves the immunodominant autoepitope on PDC-E2 (PDC-E2₂₁₂₋₂₂₆). Of pathogenic relevance is that *Helicobacter* DNA has been found in bile and liver tissue from patients with PBC, their sera containing antibodies to HELPHY, a microbe able to induce cross-reactive immune responses against gastric antigens, and hepatobiliary pathology in animals (33–36)

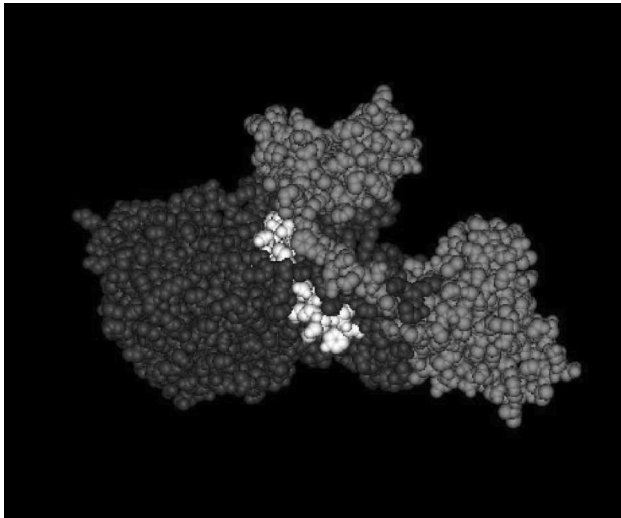


Fig. 4. Three-dimensional modelling structure of the urease beta (UREB) (dark grey) and alpha (UREA) complexes (light grey) of *Helicobacter pylori* (HELPHY) with a space fill. The PDC-E2₂₁₂₋₂₂₆ mimicking HELPHY UREB₂₂₋₃₆ sequence is shown (white). The structure was analysed with the Cn3D visualization tool. Notice the lack of exposure of the core of the peptidyl sequence to the surface of the molecule in contrast to its corners.

However, despite the universal antibody recognition of the PDC-E2₂₁₂₋₂₂₆ autoepitope, its close HELPHY UREB mimic was rarely recognized in serum samples from PBC patients, unlike other cases where microbial mimics do exhibit double- and, frequently, cross-reactivity (16).

Molecular modelling predicted that the native microbial sequence would be inaccessible to B lymphocyte receptors.

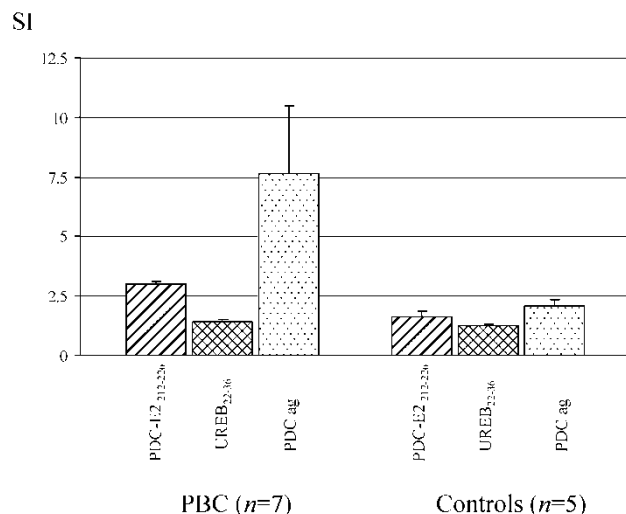


Fig. 5. Proliferative responses of peripheral blood mononuclear cells (PBMC) in 7 PBC patients and 5 pathological controls to the PDC-E2₂₁₂₋₂₂₆, HELPHY UREB₂₂₋₃₆, PDC antigen (ag). A stimulation index (SI) ≥ 2.5 was considered positive. HELPHY = *Helicobacter pylori*; UREB = urease beta.

Furthermore, the sequence would not be seen by antibody to intact PDC-E2 or its dominant epitope in the mimicking PDC-E2 sequence.

However, the synthetic peptide itself would not be in the restricted conformation existing in the native protein, and yet it does not react with antibodies recognizing the PDC-E2 peptide that it so strongly mimics. It seems, therefore, that the similarity UREB HELPHY shares with PDC-E2, is not enough for cross-recognition in spite of great amino acid homology. Compared to the human PDC-E2₂₁₂₋₂₂₆ autoepitope (KLSEGDLLAEIETDK), the HELPHY HELPHY UREB₂₂₋₃₆ mimic (RLGDTLIAEVEHDY) has a positively charged and bulky histidine (H₃₄) which would not be a conservative substitution for T₂₂₄ of PDC-E2 or any of the residues in the same relative position in any of the other microbial mimics that have shown antibody cross-reactivity to PDC-E2₂₁₂₋₂₂₆ (16), and this is evident also for the bulky and aromatic tyrosine (Y₃₆), replacing the K₂₂₆ that has been identified as an important residue in the PDC-E2 epitope (17). These differences could lead to a non-recognized conformation, particularly in a sequence that is otherwise so similar.

The above considerations would hence account for the absence of a distinct pattern of PBC-specific reactivity to HELPHY antigens in PBC patients compared to pathological and healthy controls.

In the case of T-cell epitopes, there is no conformational restriction as to which peptide may be cleaved for presentation by MHC on B-lymphocytes. However, binding for presentation may well be significantly impaired by the substitutions referred to (17). So it is not surprising that, in the present case, there is no evidence to show that the mimicking urease peptide is a CD4 epitope either. It seems therefore unlikely that HELPHY is involved in the induction of AMA antibody responses, either directly or indirectly, by a mechanism of molecular mimicry.

These clear-cut negative findings illustrate that sequence similarity does not necessarily lead to structural/conformational similarity and, hence, need not equate with actual cross-reactivity (antigenic mimicry) (7, 16, 29, 37, 38). Conversely, they suggest that cross-reactivities between autoepitopes and mimicking peptides of infecting organisms, when they are observed, are probably not trivial (7, 16), and may well have a bearing on the mechanism of disease.

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