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Characterisation of the reactivity of autoantibodies in primary biliary cirrhosis

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Autoantibodies in the sera of patients with primary biliary cirrhosis, shown previously to recognise the E2 polypeptide of the mammalian pyruvate dehydrogenase complex (PDC), have been demonstrated to react with the E2 component of PDC from bacteria (*E. coli*) and yeast (*S. cerevisiae*). Limited tryptic digestion, which cleaves E2 into well-characterised domains, followed by Western blotting indicates that the main immunodominant region of PDC E2 lies within the lipoic acid-containing domains of the polypeptide.

Primary biliary cirrhosis; Pyruvate dehydrogenase complex; Lipoic acid; Autoimmunity

1. INTRODUCTION

Primary biliary cirrhosis is a chronic, progressive cholestatic liver disease characterised by inflammatory obliteration of the intrahepatic bile ducts leading to fibrosis, cirrhosis and ultimately to liver failure [1]. PBC is the commonest indication for liver transplantation in Europe [2]. The cause remains unknown but there is considerable evidence for an autoimmune process [3], in particular a marked expansion of B lymphocyte clones producing highly specific AMA [4]. These autoantibodies are routinely demonstrated in the serum of patients with PBC by indirect immunofluorescence [5], and are directed against trypsin-sensitive antigens termed M2, associated with the inner mitochondrial membrane [6]. M2 mitochondrial antigens are known to be non-organ-specific, non-species-specific and highly conserved in evolution, with unidentified bacterial and yeast proteins cross-reacting with PBC autoantibodies [7,8].

Recently we have identified four of the M2 autoantigens as the E2 components of PDC, OGDC and BCOADC, as well as the protein X component of PDC [9,10]. Others have also reported that PDC E2 is an autoantigen [11,12].

E2 has an essential structural and catalytic role in the complexes. It forms a central symmetrical core to which E1 and E3 are bound and contains a covalently-attached lipoic acid cofactor which interacts with the active sites of each component enzyme [13]. The structure of E2 is consistent with these two properties, comprising a compact inner core domain containing the catalytic and subunit-binding sites, and one or more extended outer domains containing the lipoic acid moiety. Stretches of flexible polypeptide rich in alanine and proline residues link the different domains and contain trypsin-sensitive sites where E2 can be cleaved into separate functional domains [14,15].

In this communication we localise the major immunodominant region on the mammalian E2 polypeptide and demonstrate that the E2 com-

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Abbreviations: PBC, primary biliary cirrhosis; PDC, pyruvate dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex; BCOADC, branched-chain 2-oxo acid dehydrogenase complex; AMA, anti-mitochondrial antibodies

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ponents of yeast and bacterial PDC are recognised by PBC patients' sera.

2. MATERIALS AND METHODS

2.1. *Materials*

[2-^{14}C]Pyruvate was obtained from New England Nuclear. PDC was purified from bovine heart essentially as in [16]. E2 and protein X of PDC, which copurify with each other, were obtained by resolution of the complex using gel filtration on Superose 6 (Pharmacia) in the presence of 1 M NaCl [9]. Chicken liver H-protein was obtained from Professor Y. Motokawa (University of Tokushima, Japan), *E. coli* PDC from Professor J. Guest (University of Sheffield, England) and yeast (*S. cerevisiae*) PDC from Dr J. Gordon Lindsay (University of Glasgow, Scotland).

2.2. *Acetylation of PDC E2*

PDC E2 (2 mg/ml, containing approx. 10% of protein X) was incubated at 25°C in 50 mM potassium phosphate, 1 mM EDTA, pH 6.5, with 0.2 mg/ml PDC E1, 0.4 mM thiamine pyrophosphate, 2 mM $MgCl_2$, 0.5 mM *N*-ethylmaleimide and 0.2 mM [2-^{14}C]pyruvate (30000 cpm/nmol). Incorporation of [^{14}C]acetyl groups into protein was measured on filter paper discs as in [17]. After incubation for 30 min, dithiothreitol was added to a final concentration of 1.5 mM, and the labelled protein was digested with trypsin (1% w/w).

2.3. *SDS-PAGE and immunoblotting*

Samples of digested protein were subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) on 10% gels [18], and duplicate samples were then subjected to autoradiography, or electrophoretically transferred to nitrocellulose [19]. After blocking and washing, individual lanes were incubated with human sera at a dilution of between 1:100 and 1:1000. Detection of human IgG antibodies was by means of secondary goat anti-human IgG (γ-chain specific) peroxidase-conjugated antibodies (Sigma), with 4-chloro-1-naphthol as substrate [9].

Thirty-eight serum samples known to contain AMA directed against PDC E2 were obtained from PBC patients, and thirty-nine serum samples, negative for AMA by immunofluorescence, were obtained from patients with other chronic liver diseases and from healthy normal women. Clinical and laboratory data on these patients and controls have been given previously [9].

3. RESULTS AND DISCUSSION

When purified bovine PDC was incubated in the presence of [2-^{14}C]pyruvate and *N*-ethylmaleimide, the E2 and X components became rapidly acetylated, accompanied by a slower loss of activity of the complex due to modification by *N*-ethylmaleimide of the free-thiol group generated by the reductive acylation of the lipoic acid. However the presence of the E1 and E3 component enzymes bound to the assembled complex rendered E2 somewhat resistant to subsequent proteolysis by trypsin (not shown). Hence, [^{14}C]acetyl groups were incorporated into the purified E2-X subcomplex by incubation with [2-^{14}C]pyruvate in the presence of *N*-ethylmaleimide and approximately two pyruvate dehydrogenase (PDC E1) tetramers per E2 core. The E2 and X components became rapidly acetylated as judged by the incorporation of trichloroacetic acid-precipitable radioactivity and by SDS-PAGE and autoradiography. After labelling, the acetylated components were subjected to digestion with 1% (w/w) trypsin, the reaction being stopped by addition of excess trypsin inhibitor. Fig.1 shows the time course of this tryptic digestion. After 1–2 h digestion, two major proteolytic fragments are produced from E2 with apparent molecular masses of approximately 37 and 28 kDa as visualised by staining with Coomassie blue (fig.1a). One major radioactive band was produced corresponding to the lipoyl-containing domain with an apparent molecular mass of approx. 37 kDa (fig.1b). This is consistent with previously published data [14]. A minor radioactive band, migrating at the dye front, corresponds to the lipoyl-containing domain(s) of protein X.

When 1 μg of trypsin-cleaved E2 (and X) was immunoblotted against sera from PBC patients at a dilution of 1:1000 it was found that in all cases (38/38) the antibodies bound exclusively to the lipoyl-containing domain (fig.2) and not to any other major proteolytic fragment, indicating that the immunodominant region lies within that domain. (Reactivity to the lipoate domain of protein X could not be demonstrated using the 10% polyacrylamide gel system, due to the presence of the coloured dye. However, when a 15% gel system was employed, PBC sera were shown to cross-react with this fragment (not shown).) None of the sera from the 39 control subjects showed reactivity with any fragment.

Lipoic acid has been shown to be a mitogen, augmenting antibody responses in vitro [20] and restoring antibody responses in immunosuppressed mice [21]. The finding that the immunodominant region is located within the lipoyl-containing domain prompted us to investigate the possible role of lipoic acid in antibody binding. Firstly, in the immunoblotting procedure 1 mM lipoic acid,

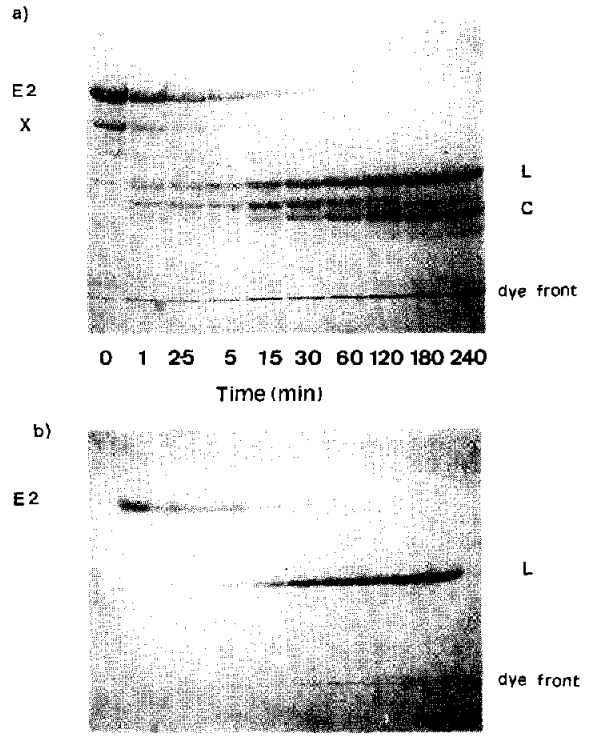

Fig.1. Time course of tryptic digestion of ^{14}C-labelled E2. E2 was acetylated in the presence of [2-^{14}C]pyruvate, *N*-ethylmaleimide and trace amounts of PDC E1, as in section 2, and dithiothreitol was then added to a final concentration of 1.5 mM. [^{14}C]E2 (2 mg/ml) was incubated with trypsin (1% w/w) at 4°C and 13 μl aliquots were taken at the times indicated and added to 28 μl soybean trypsin inhibitor (5 mg/ml) and 20 μl Laemmli's sample buffer. Samples of digested protein were subjected to SDS-PAGE on 10% gels, and subsequently autoradiographed. (a) The resultant SDS gel stained with Coomassie brilliant blue R, and (b) the corresponding autoradiograph. E2 and X are intact component enzymes; L, the lipoyl-containing domain of E2; and C, the E2 structural core domain.

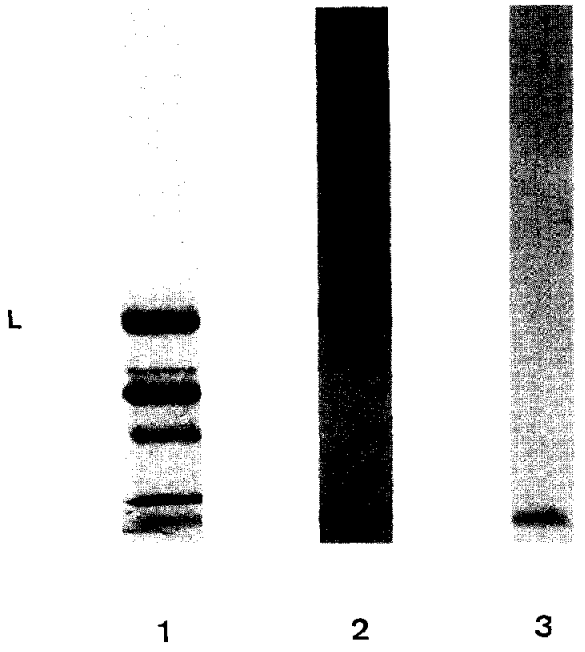

Fig.2. Reactivity of PBC sera with the domain of PDC E2 produced by tryptic digestion. ^{14}C-acetylated E2 was subjected to proteolysis by trypsin for 4 h, as in fig.1. Lanes: 1, nitrocellulose (10 μg of protein) stained with Amido black; 2,3, nitrocellulose (1 μg of protein) incubated with PBC sera at a dilution of 1 : 1000 (2), or control sera at a dilution of 1 : 100 (3), as in section 2.

1 mM lipoamide and a combination of the two were added to the incubation with sera for 2 h allowing binding, but this did not absorb out the reactivity (not shown). Secondly, we tested chicken liver H-protein, a 13.9 kDa component of the mitochondrial glycine cleavage system [22,23] for cross-reactivity in immunoblotting, as this is the only other protein, apart from the E2 components of the 2-oxo acid dehydrogenase complexes, known to contain a covalently-bound lipoic acid. There was no recognition of this protein by autoantibodies in the PBC patients' sera, even at a greatly increased loading of polypeptide (20 μg of H-protein compared to 1 μg of PDC E2). This indicates that some structural aspect unique to the lipoyl domain of the E2 components is important with respect to antigenicity, rather than reactivity being confined to recognition of lipoic acid alone. In this regard it is of interest that there is significant primary sequence homology around the lipoic-acid attachment site of mammalian and bacterial PDC E2, but less homology with the corresponding region from chicken liver H-protein [17].

In view of the known reactivity of PBC sera with yeast and bacterial proteins [7,8], and the recent identification of E2 of mammalian PDC as an M2 autoantigen, the possible cross-reactivity of patients' sera with PDC purified from *E. coli* and yeast was investigated. Fig.3 shows that the autoantibodies in PBC sera react with the E2 components of the pyruvate dehydrogenase complex from these organisms. In the case of the yeast com-

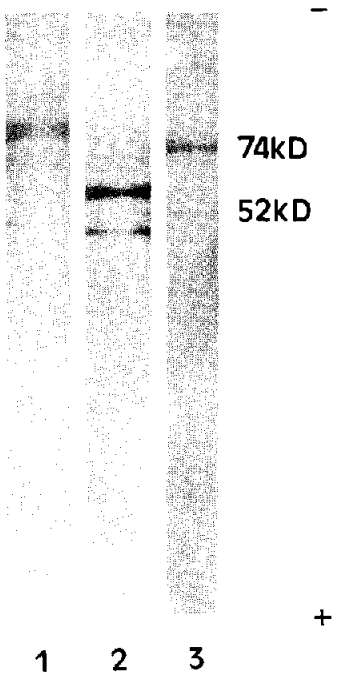

Fig.3. Reactivity of PBC sera with the E2 components of bacterial, yeast and mammalian PDC. Proteins were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with PBC sera (dilution of 1:1000) as in section 2. Lanes: 1, *E. coli* PDC (2 μg protein); 2, *S. cerevisiae* PDC (2 μg protein); 3, bovine heart PDC E2 and X (0.5 μg protein).

plex, a polypeptide of approx. 46 kDa, likely to be component X, also reacted. Similar to the mammalian complex, no reactivity was detected to E1 or E3 in either case.

Stemerowicz and colleagues [24] have recently demonstrated PBC-specific reaction patterns using Gram-negative bacteria as antigens in Western blotting, but were unable to show any reactivity against two archaebacterial strains. This finding can be explained by the fact that archaebacteria do not contain the pyruvate dehydrogenase multienzyme complex. Instead pyruvate is oxidised to acetyl-CoA by pyruvate:ferrodoxin oxidoreductase, which employs a different catalytic strategy and does not contain lipoic acid [25].

In summary we have demonstrated that anti-M2 antibodies in the sera of patients with PBC react with the E2 component of PDC, whether this polypeptide is of mammalian, bacterial or yeast origin. Our data indicate that the main immunogenic region of PDC E2 recognised by these autoantibodies lies within the lipoyl-containing domain which is located on the surface of this large multienzyme complex. Further analysis of the precise nature of the antigenic element(s) in the autoantigens may be important in understanding the immunopathogenesis of PBC and the mechanisms of self-tolerance at the molecular level.

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