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Reaction Pattern of Mitochondrial Antibodies of Primary Biliary Cirrhosis (PBC) Is Species Specific But Not Organ Specific

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Abstract

The cross reactivity of a well-characterized PBC serum was studied with mitochondria from a number of sources. These studies were to establish the nonorgan, species specificity of the reaction of PBC sera. As well as confirming some previously reported data, we have strong evidence suggesting that, in spite of species differences in M_r of the major antigenic bands, all mitochondria contain a set of common cross-reactive epitopes. The multiplicity of antigenic bands seen for mitochondria from some sources are shown to arise, in part, as proteolytically derived artefacts of bands of higher M_r, retaining some antigenic reactivity.

Key Words: PBC serum; species specificity; cross-reactive epitopes.

Introduction

Antimitochondrial antibodies (AMA) are routinely detected in sera of a large percentage of PBC patients (Doniach *et al.*, 1966). However, AMA have also been detected in other diseases such as syphilis (Wright *et al.*, 1979), drug-induced disorders (Sayers *et al.*, 1979; Homberg *et al.*, 1982), undefined collagen diseases (Labro *et al.*, 1978) and some forms of heart disease (Klein *et al.*, 1984). Nevertheless, these various AMA do not necessarily react with the same mitochondrial antigens. Thus, it is a partially diagnostic feature of PBC that the AMA detected in this disease react with trypsin-sensitive protein(s) of the inner mitochondrial membrane (Berg *et al.*, 1980, Berg *et al.*, 1982). Evidence has been put forward for the association of the PBC antigens with the mitochondrial F_1 ATPase (Sayers and Baum, 1976), but no demonstrable enzyme activity was associated with the purified antigen (Leoutsakos *et al.*,

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1982), and the binding of antibody does not seem to affect mitochondrial functions: respiration, oxidative phosphorylation, swelling, or contraction Berg *et al.*, 1969). Further investigations have shown that the PBC antigen is not a component of the F_1 ATPase complex (Sayers *et al.*, 1981) although it does copurify with the enzyme released from the inner membrane by chloroform, and it has a similar mobility to it on electrophoresis in native gels.

The AMAs present in PBC sera, on reaction with tissue sections, give a characteristic mitochondrial (M) pattern of immunofluorescence (IFL) (Meek *et al.*, 1980). The existence of other patterns of M (IFL) confirms that there must be heterogeneity of mitochondrial antigens and that (at least to some extent), AMA found in certain diseases react with different antigens (Baum and Berg, 1981). For example, it has been shown that sera from patients with cholestatic liver diseases can also cross react with the "PBC antigens" (Berg *et al.*, 1980), but AMA from other diseases, such as pseudolupus erythematosus (PLE), secondary syphilis, collagen disease (Berg and Binder, 1975), and some other autoimmune diseases do not cross react with this antigen We have now extensively documented the reaction of a large number of sera with AMA (from PBC and non-PBC patients), as monitored both by enzyme-linked immunosorbent assay (ELISA) and immunoblots, with beef heart mitochondria, beef heart submitochondrial particles (SMPs), and rat liver mitochondria (Baum and Palmer, 1985; Fusconi, Ghadiminejad, and Baum, in preparation). This has enabled us to characterize each serum with respect to ELISA titer against SMP and the patterns of bands on the different immunoblots. In this way, sera may be classified and the classification correlated with clinical history and liver pathology. This work has permitted us to select precisely defined sera to probe the molecular diversity of the antigen cross-reacting with "PBC-specific" AMA. Hence, although it has already been suggested by several workers (Lindenborn-Fotinos *et al.*, 1985; Frazer *et al.*, 1985; Baum and Palmer, 1985) that the reaction of PBC sera is species specific but not organ specific, in this study we present a more extensive account of this phenomenon, using a single, well-defined "classical" PBC serum.

Materials and Methods

Beef heart mitochondria were prepared according to the method of Beinert (1964). Liver mitochondria were prepared from various species according to the method of Massari *et al.* (1972). Mitochondria were also prepared from heart, spleen, kidney, brain, and lungs from rat, rabbit, and guinea pig using essentially the same method (Massari *et al.*, 1972), with slight modifications. For the preparation of mitochondria from brown adipose

tissue and white fat, essentially the same techniques as above were used except that the tissue homogenates were first passed through a layer of nylon gauze (pore size 0.1 mm in size) and centrifuged at 8500 *g* for 10 min. The hard packed fat layer (on top) and the supernatant were discarded, and the pellet of cell debris and the mitochondria were then treated as before. Rat tissue mitochondria were also prepared in the presence of a proteolytic enzyme inhibitor, phenylmethylsulfonyl fluoride. This inhibitor (0.1%) was included in the isolation buffer, 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, and 5 mM MOPS-KOH, pH 7.4, using the same methods described above.

Saccharomyces cerevisae (yeast) were grown in liquid media containing 1% yeast extract and 2% glucose at 30°C for 48 hr with gentle agitation. The culture medium cell suspension was centrifuged at 800 *g* for 10 min and the cell pellet was washed twice in cold isolation buffer, 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, and 5 mM MOPS. The cells were homogenized using an MSK Braun cell homogenizer with balletini beads of 0.25–0.30 mm size. This homogenizer has a disruption efficiency of over 85%. The cell homogenate was passed through a double layer of nylon gauze (0.1 mm pore size), and the cell homogenate, free from balletini beads, was then treated similarly as before, but using this isolation medium.

Human liver biopsies were homogenized using a Potter homogenizer in minimum volume of buffer, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4. Rat hepatoma cells were cultured as previously described (Ding *et al.*, 1981). The cells were homogenized using a Potter homogenizer for 35 strokes. The cell homogenate was then used for mitochondrial preparation (Massari *et al.*, 1972). Mitochondria were also prepared from potato according to the method of Banner (1967), and from *Arum maculatum* spadix according to the method of Cammack and Palmer (1977). The protein content of samples was determined according to the method of Bradford (1976). The mitochondrial fractions were tested for enrichment of mitochondria by assaying for succinate dehydrogenase according to the method of Jenkins and Peters (1978).

PBC Serum

A large quantity of PBC serum from one patient was obtained when plasma exchange was performed. The patient was clinically classed to be in stage 4 of the disease at the time of plasma exchange.

Immunological Techniques

The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-page) according to the published procedure (Laemmli,

1970; Laemmli and Favre, 1973). Electrophoresis was carried out at a constant current of 50 mA, while the gels were being cooled by electric fans, until the dye front had migrated 10 cm in the separating gel (10% total acrylamide, 0.375 M Tris-HCl, pH 8.8, 2 mM EDTA, 0.1% SDS). Prior to electrophoresis, the samples were diluted in sample buffer (final concentration 0.05 M Tris-HCl, pH 6.8, 2 mM EDTA, 2% SDS, 50 mM dithiothreitol, 0.001% bromophenol blue) to a protein concentration of 1 mg/ml (where possible) and were incubated at 37°C for 1 hr. The gel was electroblotted onto a sheet of nitrocellulose (pore size 0.45 μm, Sartorious Membrane Ltd.), according to the method of Towbin *et al.* (1979). The antibody–antigen complexes were detected by the binding of iodinated second antibody (rabbit antihuman antibodies from DAKO were iodinated by the chloramine T method; Hunter and Greenwood, 1962; Greenwood *et al.*, 1963). The dried nitrocellulose sheet was exposed for 10 days to double-sided X-ray film (Scopix CR3, Agfa, Gevaert, London) in a cassette (X-ray Inspection Equipment Ltd, London).

To assess the M_r of the bands detected, on one lane of the gel, molecular weight standards (Sigma chemicals, USA), consisting of phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soyabean trypsin inhibitor (20.1,000), and lactalbumin (14.4,000) were used. These were treated in the same way as the mitochondrial proteins and were loaded on the gel. After immunoblotting, the strips with the standard proteins were cut to size and stained for protein with amido black, 0.1% in 25% isopropanol and 10% acetic acid, and destained with the same solution without the dye. The other section of the nitrocellulose sheets with the mitochondrial proteins were developed as above and the M_r of the antigenic bands were determined.

Pre-absorption of Sera

To absorb out the AMA from the PBC sera, 50 μl of the sera was incubated with 100 μl of mitochondria with a protein concentration of 10–15 mg/ml, diluted to 1 ml in an Eppendorf tube with incubation buffer, 3% hemoglobin, and 5% v/v inactivated horse serum in saline (20 mM Tris-HCl, pH 7.5, containing 9% sodium chloride) at room temperature for 2 hr. The mixture was shaken at regular intervals to ensure efficient antigen–antibody binding. The antigen–antibody complexes and the mitochondria were precipitated by high-speed centrifugation in a microcentrifuge for 5 min. This process may be repeated several times for maximum removal of AMA from the sera. Finally, the supernatant was diluted to 5 ml with the incubation buffer which was used for the detection of any residual specific binding to mitochondrial proteins on a strip of nitrocellulose, cut to size.

Results and Discussion

Cross Reactivity of PBC Sera with Beef Heart Mitochondrial Antigen(s)

To provide the standard by which to compare antigenic species from various sources, the cross reactivity of the selected, stock PBC serum was first studied with the well-characterized beef heart mitochondria, using the sensitive immunoblotting technique. The pattern and the number of bands produced by the cross reactivity of this serum were compared with the reactivity of other sera held in our laboratory from PBC patients with similar clinical backgrounds. The reactivity was found to be typical, with respect to the number and the pattern of bands detected on the immunoblot, for PBC sera from patients at stage 4 of the disease. These studies revealed 5 antigenic species, one of very high molecular weight (M_r), and two with a lower M_r than the two main antigenic species of M_r 74,000 and 68,000 (Fig. 1, track 1). However, the number of antigenic species seemed to vary between different beef heart mitochondrial preparations, and these differences became marked on prolonged storage. It can be seen from Fig. 1, track 2, that in a sample thawed several times there are more lower M_r antigenic species than previously observed, and the band of very high M_r (approximately 150,000) was not detected, nor was the major band of the lowest M_r. By contrast, the second antigenic species, of M_r 68,000, was now more intensely expressed. These observations seem to confirm the susceptibility of the PBC antigens to proteolytic breakdown (Berg *et al.*, 1969), and

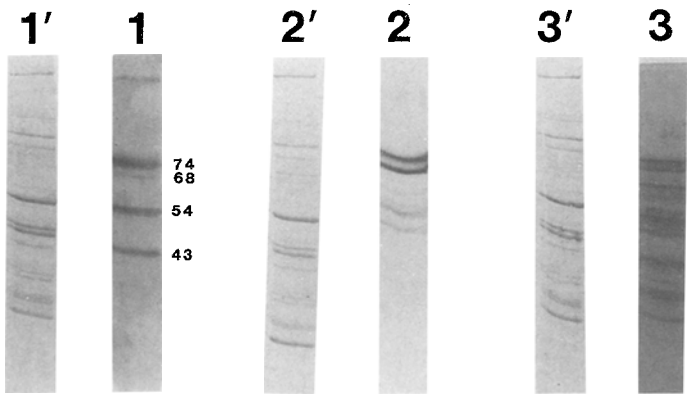

Fig. 1. Reaction of PBC serum with beef heart mitochondria. (1) Antigenic species of freshly prepared mitochondria detected by immunoblotting; (2) antigens detected in sample following stroage at −20°C and several thawings; (3) antigenic bands detected when mitochondria were allowed to stand at room temperature overnight. (1′, 2′, 3′) Coomassie blue staining of corresponding gels prior to immunoblotting.

since the breakdown of one band seems to enhance another, it might be suggested that the various bands detected even in fresh preparations derive from a common species of high M_r either by proteolytic processing or by SDS-mediated depolymerization. These finding are compatible with those of Ben-Yoseph *et al.* (1974). However, the situation is certainly more complex than is implied by this explanation, since different PBC sera may develop different members of the bands of a single mitochondrial sample (Baum and Palmer, 1985).

When mitochondrial samples were allowed to equilibrate to room temperature and stand overnight before sample treatment and gel electrophoresis (Fig. 1, track 3), then several species of even lower M_r were detected, thus supporting the previous conclusion in regard to the pattern seen with a single serum.

Interestingly, comparison of the pattern of staining with Coomassie Blue of the three preparations separated on a 10% SDS-PAGE shows very little difference in the number of proteins detected on the gel (Fig. 1, tracks 1, 2, and 3). These findings further confirm the insignificant amount of the PBC antigens present in mitochondria. Despite the differences in the number of antigenic species detected by a single standard serum, most probably due to proteolytic breakdown, beef heart mitochondria were used as reference markers for the antigenic species of other mitochondria, since the main antigenic species were easily identifiable.

Non-Organ Specificity of PBC Antigen(s)

Reaction of the PBC serum with mitochondria obtained from different organs of rat showed a slight variation in the number of antigenic species detected. However, the main antigenic species and many of the minor bands show great similarity in M_r (Fig. 2). These observations further confirm the reported non-organ specificity of the reaction of PBC sera with mitochondrial antigens.

The main antigenic species of rat organ mitochondria (M_r 63,000) can be seen to differ from that observed in the control track (beef heart mitochondria, M_r 74,000). However, two of the minor antigenic species from the control track seem to be of the same M_r as the two corresponding bands seen in mitochondria from rat organs.

The susceptibility of the PBC antigens to proteolytic breakdown observed in beef heart mitochondria prompted an investigation into the susceptibility of PBC antigens in other mitochondrial sources. The same rat organ mitochondria were prepared in the presence of the proteolytic enzyme inhibitor, phenylmethylsulfonyl fluoride (PMSF). Figure 3a illustrates that in the presence of the inhibitor only three antigenic species were detected in all eight cases, and that the first antigenic band (of approximately M_r 63,000)

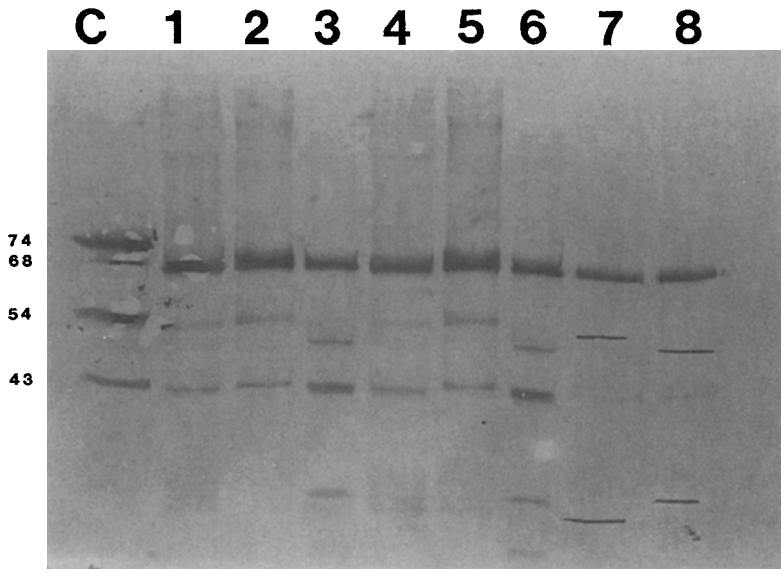

Fig. 2. Reaction of PBC serum against immunoblots of rat organ mitochondria. Mitochondria from beef heart (C) was used as standard for the PBC-reactive mitochondrial antigens from heart (1), liver (2), spleen (3), kidney (4), lungs (5), brain (6), brown fat (7), and white fat (8).

exhibited maximum intensity. However, the two minor bands (lower M_r bands were found to be of the same M_r as the two corresponding minor bands in beef heart. To investigate the extent of which these antigens were susceptible to proteolytic breakdown, earlier samples (as used in Fig. 2) were intentionally incubated under conditions favorable for proteolysis, i.e., they were allowed to stand at room temperature for 36 hr (Fig. 3B). Although, in each case, equal amounts of protein were applied on the gels (Fig. 3A and B), the intensity of the antigenic bands was seen to decrease to an extent which could not be accounted for by the increased number of bands detected (Fig. 3B). These findings not only confirm the susceptibility of these antigens to proteolysis, but suggest that the degraded molecules may no longer possess the full complement of the antigenic determinants.

Species Specificity of PBC Antigens

In order to investigate the cross reactivity of the PBC serum with mitochondria from different species, it was necessary to establish that the antigens detected in mitochondria from organs of one species had the same antibody specificity as did antigens detected in mitochondria from organs of another species. To investigate this, the PBC serum was pre-absorbed with mitochondria from one source, and its reaction with mitochondria from

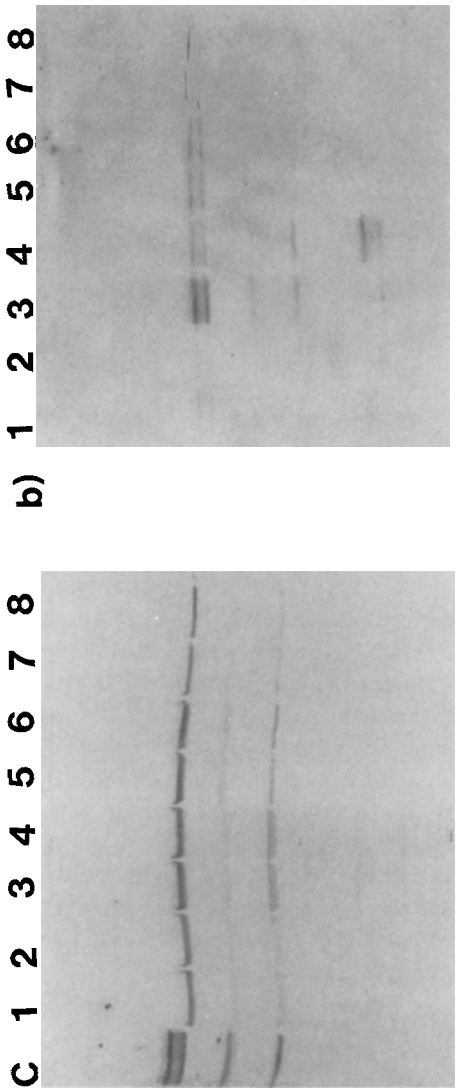

Fig. 3. Effect of endogenous proteolysis on the reaction of PBC serum against immunoblots of rat organ mitochondria. The rat organ mitochondria were prepared in the presence of a proteolytic inhibitor, phenylmethylsulfonyl fluoride, PMSF (a) or they were prepared in the absence of the inhibitor and were allowed to stand at room temperature for 36 hr (b). Mitochondria were prepared from the following organs: heart (1), liver (2), spleen (3), kidney (4), lungs (5), brain (6), brown fat (7), and white fat (8). Lane C on immunoblot (a) is beef heart mitochondria as standard.

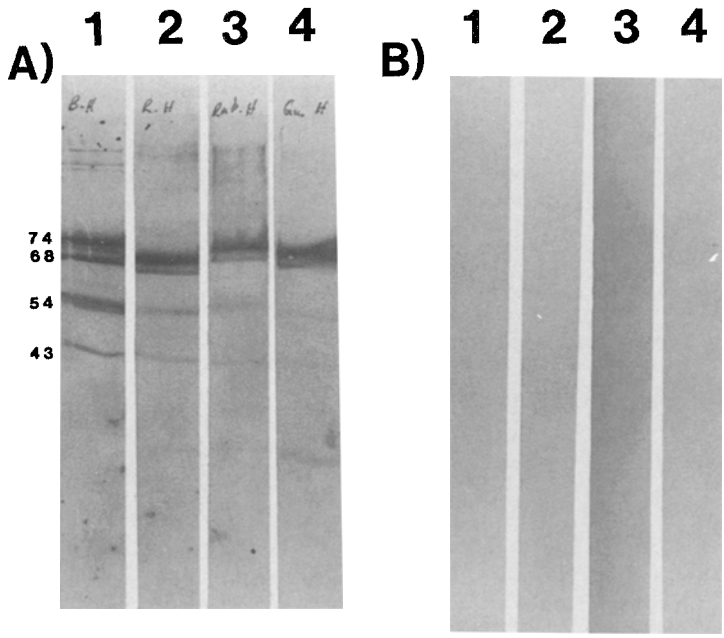

Fig. 4. Reaction of PBC serum pre-absorbed with mitochondria from beef heart. Mitochondria from beef heart (1), rat heart (2), rabbit heart (3), and guinea pig heart (4) were subjected to electrophoresis (10% SDS-PAGE), electroblotted onto nitrocellulose, and labelled with PBC serum, either untreated (A) or absorbed with beef heart mitochondria (B).

another source was then assessed. Figure 4A shows the normal reactivity of the PBC serum with mitochondria from four different sources. As illustrated in Fig. 4B, pre-absorption of the serum with mitochondria from beef heart abolished all reactivity. Identical results were obtained following pre-absorption of serum with mitochondria from rat liver or rabbit kidney. A similar cross reactivity study was made between yeast mitochondria and beef heart mitochondria, (Fig. 5). In this case relatively large amounts of yeast mitochondria were required totally to absorb out the AMA reacting with beef heart mitochondria, although (not shown) sera absorbed with beef heart mitochondria did not react at all with those of yeast. This may simply be due to the organization of the antigenic determinants in yeast mitochondria prior to denaturation with SDS. It is nevertheless particularly interesting that the two PBC-reactive antigenic species of yeast mitochondria (Ghadiminejad and Baum, 1985b, Uzoegwu *et al.*, 1984) seem to be able to absorb from PBC sera the antibodies cross-reacting with all the antigenic species detected in beef heart mitochondria. This suggests that these two antigenic bands contain all the antigenic determinants carried by the antigenic species detected in beef heart mitochondria.

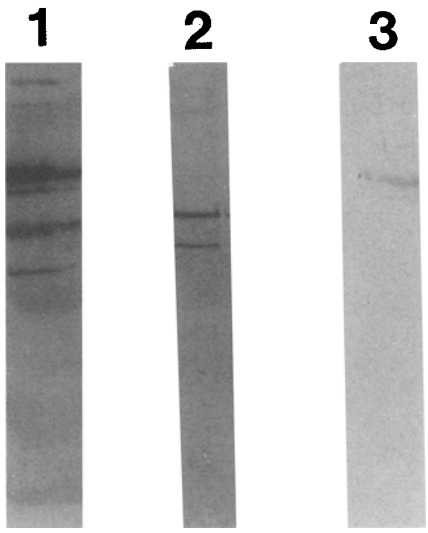

Fig. 5. Reaction of PBC serum pre-absorbed with yeast mitochondria. (1) Antigenic species of freshly prepared beef heart mitochondria detected by immunoblotting; (2) antigenic species of freshly prepared yeast mitochondria; (3) reaction with beef heart mitochondria of PBC serum pre-absorbed with yeast mitochondria.

The reaction of the PBC serum with rabbit organ mitochondria and guinea pig organ mitochondria further confirmed the non-organ specificity of the PBC antigens with respect to the main antigenic bands (not shown). However, to compare the differences in the M_r of the major antigenic bands, a selection of samples was tested on the immunoblot of one gel. The differences observed were rather small and, for better resolution, the samples were run on a 5–15% gradient SDS-polyacrylamide gel (Fig. 6). As can be seen from Fig. 6B, the M_r's of the major antigenic species from heart and liver of any one species are very similar. The species specificity is clearly illustrated by the relatively large differences observed in the M_r of the major band in yeast mitochondria compared to that of, say, rabbit. To further illustrate the species specificity of the PBC antigens, mitochondria from potato and arum and unfractionated homogenates of snap-frozen biopsies of human liver (from PBC and non-PBC patients) were tested (Fig. 7). These findings clearly establish the species specificity of PBC antigens. (Note that the major antigens of mitochondria from rat hepatoma are similar to those for rat liver mitochondria.) However, it should be pointed out that the two high-molecular-weight bands observed from the reaction of PBC serum with both human liver homogenates (PBC and non-PBC) are not PBC-specific since similar bands were observed from the reaction of normal

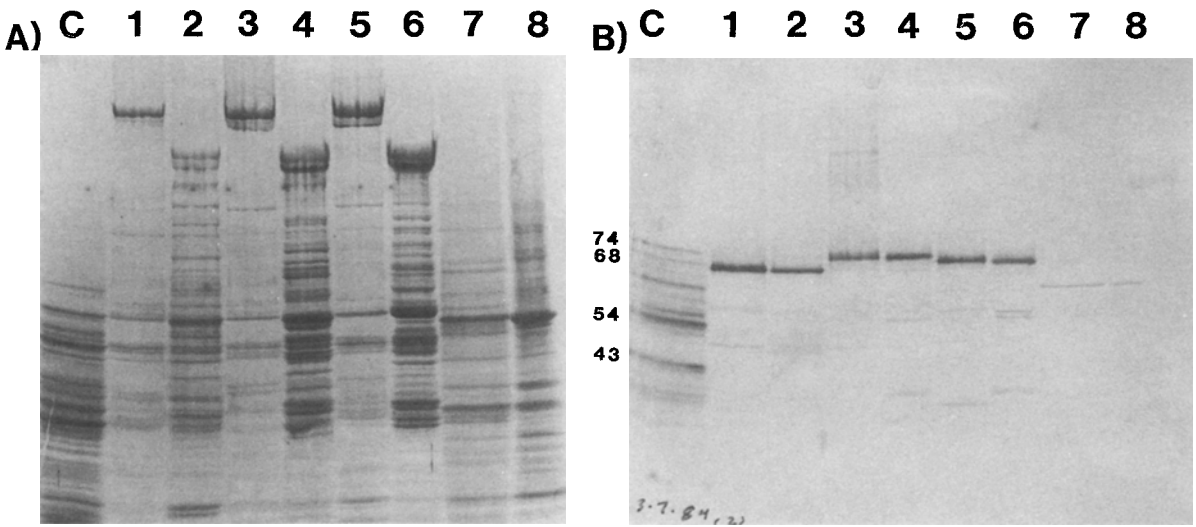

Fig. 6. Comparison of mitochondrial antigenic species on a 5–15% gradient gel. Mitochondria from beef heart (C, aged sample), rat liver (1), rat heart (2), rabbit liver (3), rabbit heart (4), guinea pig liver (5), guinea pig heart (6), yeast mitochondria (7), and premitochondrial pellet from wild type yeast (8) were subjected to 5–15% polyacrylamide gel electrophoresis and electroblotted. The immunoblot was stained with PBC sera (**B**) and the gel with Coomassie Blue (A).

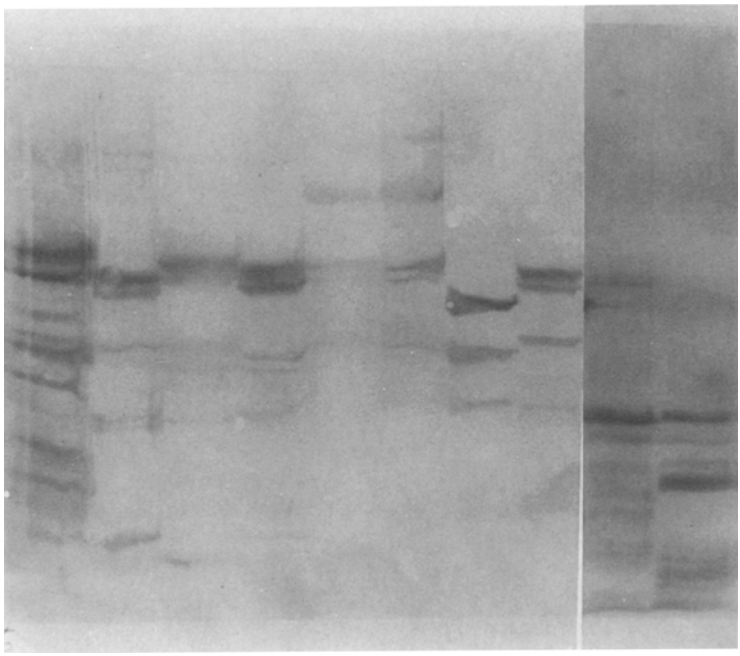

Fig. 7. Comparison of PBC antigens from a number of sources. The ten lanes show the PBC-reactive antigens in beef heart, rat liver, rabbit liver and guinea pig liver mitochondria, human liver homogenate (non-PBC; 50 μg of protein applied), human liver homogenate (PBC, 80 μg of protein applied), yeast (wild type), rat hepatoma, potato, and arum mitochondria respectively.

sera with these preparations. (The higher M_r band is not readily apparent for the normal liver sample in this figure, but this is because of the small amount of protein loaded in this case.) These bands are thought to be due to different blood grouping, since the reaction of normal sera with the same blood grouping as that of the biopsy donor in each case failed to produce these bands (results not presented). Interestingly, the liver homogenates from PBC and non-PBC patients expressed similar antigenic species with regard to M_r and with the same intensity of the bands (with respect to the amount of protein applied).

The beef heart, rat, rabbit, and guinea pig liver and yeast mitochondrial samples used in Fig. 7 were those used in previous experiments, except that the samples had been frozen and thawed several times. It is interesting to note that this process considerably enhanced the apparant proteolytic degradation of the PBC antigens. Beef heart mitochondrial antigens in particular seem to have been extensively degraded, whereas in liver mitochondria from rat, rabbit, guinea pig, the most prominent observation is that the first main antigenic band is now detected as two close but distinct antigenic species. In

the case of yeast mitochondrial antigens, proteolysis seems to have resulted in a third, lower M_r antigenic band. These findings not only reconfirm the susceptibility of the PBC antigens to proteolysis, but might suggest that each band detected is composed of several antigenic species of similar M_r but differing in susceptibility to degradation by endogenous proteases. This possibility is strengthened by work carried out in our laboratory using two-dimensional gel electrophoresis, where the major antigenic bands of fresh mitochondria are seen to separate to several antigenic species exhibiting different isoelectric points (M. Wong, personal communication). Finally, although the antigenic species detected in mitochondria from the two plant species are of much lower M_r compared with their mammalian counterparts, they are able to absorb out from the PBC serum the AMAs cross-reacting with beef heart mitochondria (results not shown). The reverse was also found to be true. This in conjunction with earlier pre-absorption studies has led us to conclude that the differences in M_r's of the major antigenic bands detected in mitochondria from different species might be due to species-specific patterns of processing of a common precursor molecule (Ghadiminejad and Baum, 1985a). This concept is supported by the observation that many of the minor antigenic species detected in mitochondria from all the sources studied seem to be very similar in M_r. However, as mentioned earlier, such an hypothesis does not account for the fact that sera from some patients do not react with all of the bands developed by other sera with a given mitochondrial preparation. Also it must be borne in mind that polyclonal antibodies of several immunoglobulin classes, as are present in PBC, might have a spectrum of epitopic specificities and avidities; and the fact that PBC antigens are glycoproteins (Ghadiminejad and Baum, 1985b) together with the multiplicity of antigenic species present in a single band (see above), further complicates the situation.

Nevertheless, it does seem likely, from the work presented here, that, whatever the species of organ studied, the AMA of PBC are reacting predominantly with a single class of glycoproteins, or their degradation products retaining the common epitope. The nature of this ubiquitous class of protein has still to be established.

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