

MITOCHONDRIAL ANTIBODIES IN PRIMARY BILIARY CIRRHOSIS

I. LOCALIZATION OF THE ANTIGEN TO MITOCHONDRIAL MEMBRANES

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PLATES 26 AND 27

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A number of reports have described complement fixation reactions of sera from patients with primary biliary cirrhosis (PBC) using subcellular tissue fractions (1-3). Subsequently it was shown that almost all these patients give cytoplasmic staining in the immunofluorescent test, reacting preferentially with cells rich in mitochondria (4-6). These antibodies are consistently absent in extrahepatic biliary obstruction and their detection has proved of clinical importance in that surgical exploration can be avoided in cases of jaundice due to primary biliary cirrhosis. Further studies are now reported demonstrating that these antibodies are directed specifically against mitochondrial membranes.

Materials and Methods

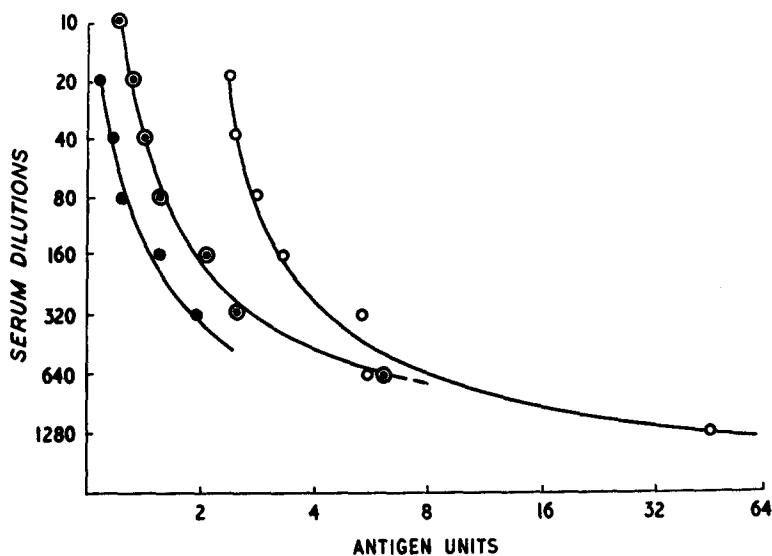
Patients.—34 sera were selected from known cases of primary biliary cirrhosis on the basis of the characteristic fluorescent pattern, a high titer in the complement fixation test with rat liver homogenate, and absence of unwanted serological reactions including antinuclear and rheumatoid factors, smooth muscle fluorescence, or organ-specific thyroid and gastric antibodies.

Quantitative Complement Fixation Method (CFT).—The method of Rapport and Graf (7) was used as applied to the study of microsomal antigens (8, 9). The Ca/Mg CFT buffer tablets (Oxoid Ltd., London) were made up with addition of 0.1% bovine serum albumin. Sheep cells (Burroughs Wellcome & Co., London, England) in Alsever's solution were allowed to stabilize for 1 wk, then used for 2-4 wk. A 6% suspension was sensitized with an equal volume of hemolysin and titrated with excess complement. The titer of guinea-pig complement (BW dried preserved) was established by the method of Osler et al. (10) and the 50% hemolytic unit ($C'H_{50}$) calculated by interpolation from the plot of percentage lysis on a "logit" scale against concentration of complement on a "log" scale.

For the assay of antigen, 0.1 ml of antigen dilution was mixed with 0.1 ml of the standard PBC serum dilution and 3 $C'H_{50}$ units of complement, and made up to 1.2 ml with Ca/Mg buffer. All the reagents were kept in ice during the pipetting to minimize loss of C'. The system was incubated at 37° for 1 hr, 0.3 ml of sensitized indicator cells was added and incubation

continued for a further 1 hr with frequent shaking. The tubes were spun at 4° and the degree of lysis determined by the absorption at $541\text{ m}\mu$. Serial dilutions of each antigen fraction were assayed and the amount giving 50% lysis was defined as one unit. Antigen and serum controls contained $1.5\text{ C}'\text{H}_{50}$. With a given series of reagents the assay was reproducible to within 10%.

To select the standard serum for mitochondrial antigen assay, serial dilutions of several PBC sera were tested against increasing amounts of mitochondria and isofixation curves constructed by estimating the level of antigen required to fix 2 out of 3 $\text{C}'\text{H}_{50}$ for each dilution of serum studied. The three isofixation curves shown in Text-fig. 1 were obtained with three



TEXT-FIG. 1. Isofixation curves obtained with three primary biliary cirrhosis sera using a mitochondrial preparation as antigen. Each point on the curves represents the amount of antigen fixing two out of three $\text{C}'\text{H}_{50}$ units of complement. ●, Serum from patient L. H. selected as standard.

PBC sera and a single mitochondrial preparation. The titers were between 640 and 1280. Differences between the curves in antibody excess are presumably related to the number of antigenic sites participating in the reaction of mitochondria with different PBC sera. The serum finally selected (L.H.) gave a nearly vertical isofixation curve in antibody excess and detected antigen at a sensitive level. It was obtained from a female patient aged 51 yr who had had obstructive jaundice for 3 yr and in whom primary biliary cirrhosis had been proven by wedge biopsy obtained at exploratory laparotomy. This serum was used throughout at a dilution of 1:20 for the quantitative CFT and for studying the distribution of antigen in subcellular fractions.

Immunofluorescence Tests.—The IgG of L.H. standard PBC serum isolated by chromatography on DEAE cellulose in 0.02 M phosphate buffer pH 7.0 was conjugated directly with fluorescein isothiocyanate using a ratio of 0.05 mg to 1 mg globulin. Unconjugated dye was removed on a Deacidite FF (Permutit Co. Ltd., London) column and the conjugate absorbed with pig liver powder (Burroughs Wellcome). The direct PBC conjugate was diluted 1:3 and

applied to unfixed cryostat sections of various rat and human tissues. For the double layer test, rabbit anti-human globulin sera were used, as well as conjugates of specific anti-IgG, IgA, and IgM. The antibodies could be demonstrated in the three main classes of the patient's immunoglobulins.

Absorption of Mitochondrial Fluorescence.—For this 0.02 ml of L.H. serum and other PBC sera were successively absorbed with two 0.18 ml aliquots of concentrated mitochondrial suspension, giving a final dilution of 1:100. After standing overnight at 4° the mixture was spun and the supernatant tested by the double layer fluorescent test. Unabsorbed serum aliquots similarly diluted with saline were set up as controls.

Tissue Fractionation Procedure.—Fresh tissues were taken from 3-month-old Wistar rats weighing 150–200 g killed by decapitation. The method of de Duve et al. (11) was used with some modifications. 25–30 g of fresh rat liver obtained from animals starved for 14–20 hr, was washed in cold saline, minced with scissors, and disrupted in three volumes of 0.25 M sucrose, using a glass homogenizer with Teflon pestle (No. C13916, Arthur H. Thomas Co., Philadelphia, Pa.) having a clearance of 0.15–0.22 mm. To standardize the procedure, only two passes were made. The homogenate was spun at 600 g for 10 min and the nuclear pellet washed twice with three volumes of sucrose, rehomogenized as before and spun at 260 g, the washings being added to the original supernatant to give a final volume of 10 ml/g of liver. This cytoplasmic extract was respun at 600 g to remove red cells and unwanted aggregates, and then at 8000 g for 10 min. The supernatant and the fluffy layer were each carefully sucked off and kept separately; the surface of the mitochondrial pellet was washed with sucrose and the pellet rehomogenized and suspended in the original volume. This was repeated twice, halving the volume at each wash. The washed pellet, which was light brown in color, was resuspended in a small volume to give a concentration of 50–70 mg protein per ml and kept in 1 ml aliquots at –20°. This represented the “purified” mitochondrial preparation (MI). The mitochondrial washings were respun at 8000 g for 10 min and the pellets (MII), which were of a pink color, were added to the main fraction in some experiments to increase the yield. The fluffy layer overlying the mitochondrial pellet MII was removed and kept separately.

When microsomal fractions were required, the supernatants were spun at 26,000 g for 30 min (Spinco model L; rotor no. 40) and the pellet washed twice. This pellet contained lysosomes, heavy microsomes, and light or broken up mitochondria. The supernatant from this fraction was spun at 105,000 g for 1 hr and the microsomal pellet washed twice.

Purified lysosomal preparations were obtained from the livers of rats given 200 mg of Triton WR 1339 (Rohm & Haas Co., Philadelphia, Pa.) intravenously 4 days previously (12). A 1:10 homogenate was prepared as before and spun at 37,000 g for 8 min to obtain the mitochondria-lysosome fraction. The fluffy layer was carefully removed. The pellets were washed in one-third of the previous volume and the final pellet was resuspended in 10 ml of 45% w/w sucrose and placed at the bottom of Rotor SW 39 tubes (Spinco model L2), the gradient being layered on top with densities from 1.21 to 1.06 as shown in Text-fig. 5. After spinning at 100,000 g for 1 hr the lysosomes gathered at the two interfaces of the 1.14 layer; mitochondria and microbodies remained in the pellet M₁ and in the fraction M₂ which gathered at the lower interface of the 1.15 sucrose layer. Homogenates of kidney, heart, spleen, and thymus were prepared in the same way as for liver. Cardiac muscle required more prolonged homogenization.

Fragmentation of Mitochondria.—Freshly prepared purified mitochondria were submitted to the following treatments, the final protein concentration being adjusted to 16 mg/ml. After spinning at 26,000 g for 15 min and then at 150,000 g for 80 min they were tested by CFT on the same day. *Ultrasound:* 2 ml samples were sonicated for 2 min as described by Roitt et al. (8). *Hypotonicity:* 6 ml of 10 mM phosphate buffer pH 7.2 were added to 2 ml of mitochondrial preparation and stirred for 60 min at 4°. *Hexane:* mitochondria were mixed with an equal volume of hexane and shaken in a Whirlimixer (Scientific Industries Inter-

national, Queens Village, N. Y.) for 30 sec. *Lysolecithin*: an equal volume of 1% lysolecithin (Koch-Light Lab.) in CFT buffer was added to mitochondria and incubated for 10 min at room temperature. 0.5% lysolecithin caused partial lysis of the red blood cells in the CFT. As a result of this, the antigen remaining in the final 150,000 g supernatant could not be assayed directly but was estimated by difference from the activity recovered in the two pellets.

In another experiment a purified mitochondrial preparation was suspended in 0.75 mM phosphate buffer pH 7.2 for 20 min at 4° and centrifuged at 105,000 g for 10 min; the treatment was repeated on the pellet and the combined supernatants spun at 150,000 g for 120 min. The final pellet was examined by electron microscopy.

Marker Enzyme Tests.—Succinic dehydrogenase was determined by the method of Slater and Planterose (13). Glucose 6-phosphatase and acid phosphatase were estimated according to de Duve et al. (11).

Histochemical Tests.—Cryostat sections were fixed for 10 min in 10% formol-saline and stained for succinic dehydrogenase by the method of Nachlas et al. (14). In each case the adjoining unfixed serial section was treated with the fluorescent standard PBC conjugate for comparison of the staining patterns.

Protein Estimations.—The biuret method was employed for tissue fractions and at low concentrations the method of Lowry et al. (15) was used with a BSA standard.

RESULTS

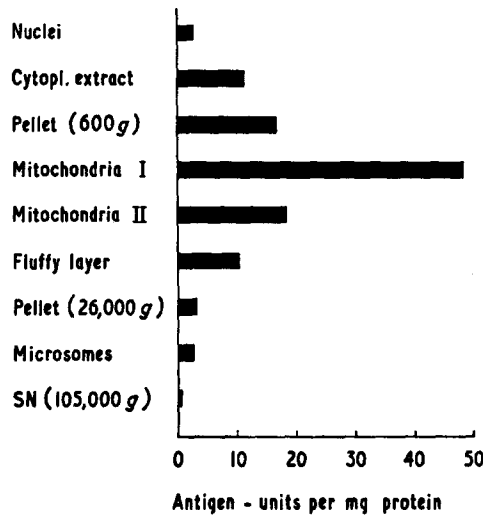
Localization of Complement-Fixing Antigen in Mitochondria

Distribution of CF Antigen in Subcellular Fractions.—The distribution of antigen activity was studied in liver subcellular fractions obtained by differential centrifugation, using the standard (L.H.) PBC serum. In 12 experiments between 50% and 75% of the antigen was recovered in the crude mitochondrial fraction. The remaining antigenic activity was mainly in the nuclear fraction and in the 26,000 g fluffy layer. Microsomal pellets and supernatant retained not more than 4% of the activity. Mitochondrial preparations usually contained approximately 2000–3000 antigen units/ml. The specific activity of the combined mitochondrial fractions M1 and M2 was 34 ± 3 (SD) units/mg protein but this could be increased up to 53 ± 9 units/mg in purified preparations. The mitochondria had the highest specific activity of any subcellular fraction (Text-fig. 2). A purified preparation was used for the electron micrograph shown in Fig. 3 which confirms the low degree of contamination with other organelles although some contamination with lysosomes and membranous elements was seen in another zone of the mitochondrial pellet.

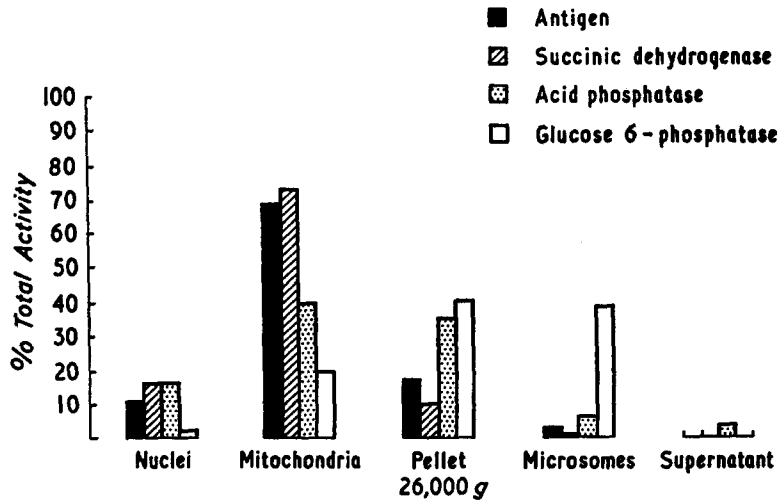
34 proven PBC cases were tested in the quantitative CFT with rat liver nuclei, mitochondria, lysosomes, microsomes, and 105,000 g supernatant concentrated threefold. The sera were selected for their high titers with whole homogenate and were tested at a dilution of 1:20. All these sera reacted strongly with the mitochondrial pellet. A few gave weak reactions with other subcellular fractions but these reactions could be eliminated by retesting with purified preparations and were therefore thought to be due to contamination with mitochondria or fragments.

Correlation of CF Antigen with Marker Enzymes.—To identify the contri-

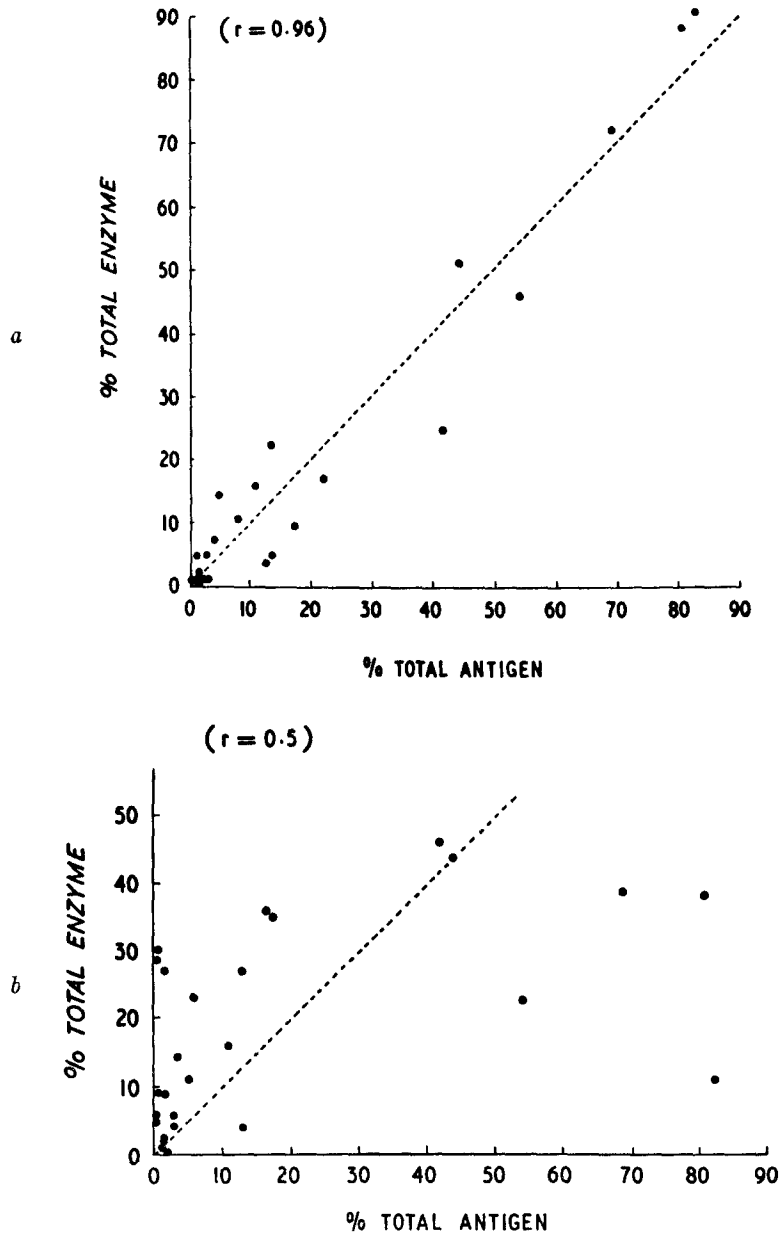
bution of different organelles within the subcellular fractions, marker enzymes were estimated. Succinic dehydrogenase was used as mitochondrial marker, acid phosphatase for lysosomes and glucose 6-phosphatase for microsomes. Text-fig. 3 shows a representative crude fractionation. It can be seen that suc-



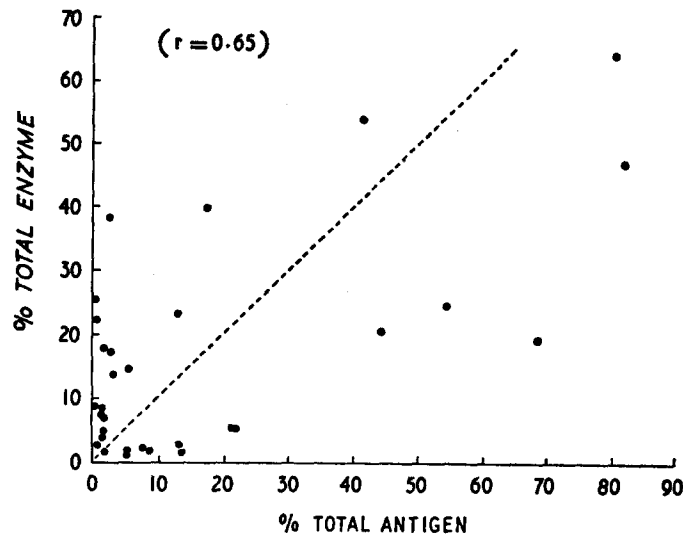
TEXT-FIG. 2. Specific activity of CF antigen in subcellular liver fractions obtained by differential centrifugation as described under "Methods and Materials."



TEXT-FIG. 3. Distribution of CF antigen and marker enzymes in subcellular fractions of rat liver obtained by differential centrifugation. The mitochondrial fraction corresponded with MI and MII as described in the "Methods and Materials" section.



TEXT-FIG. 4. Correlation of CF antigen content with marker enzyme activity in sub-cellular liver fractions obtained in six separate experiments: (a) succinic dehydrogenase (mitochondrial marker); (b) acid phosphatase (lysosomes); (c) glucose 6-phosphatase (microsomes).



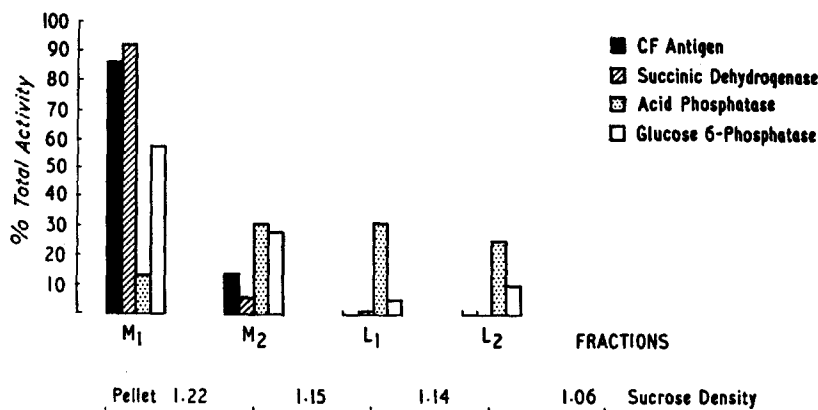
TEXT-FIG. 4 c.

cinic dehydrogenase activity paralleled the CF activity in each fraction and was maximal in the mitochondrial pellet. This pellet still contained lysosomes and microsomes as shown by their enzyme activities. The three marker enzymes were determined in a number of different fractions obtained in six separate experiments by differential and density gradient centrifugation, and correlated with the CF activity of each fraction. The correlation coefficient for succinic dehydrogenase was 0.96 (Text-fig. 4 a), confirming the mitochondrial location of the antigen. Lower correlation coefficients of 0.5 and 0.65 were obtained for acid phosphatase and glucose 6-phosphatase, respectively (Text-figs. 4 b and c).

Analysis of Lysosomes.—To confirm that lysosomes are not involved in the reaction with primary biliary cirrhosis sera, purified lysosomes were prepared from the livers of rats pretreated with Triton. This detergent is taken up by the liver lysosomes and decreases their specific gravity, making it easier to separate them from mitochondria (11). The four fractions M_1 , M_2 , L_1 , and L_2 , obtained by centrifugation of the large granule pellet on the sucrose gradient, were tested for CF activity and for marker enzymes (Text-fig. 5). Neither of the lysosomal fractions contained antigen or appreciable succinic dehydrogenase activity whereas the pellet M_1 containing the bulk of the mitochondria represented 85% of the antigenic activity and 90% of the succinic dehydrogenase. The other mitochondrial fraction M_2 accounted for the remainder of the antigen and succinic dehydrogenase, though it also contained 30% of the acid phosphatase activity owing to contamination with lysosomes.

Correlation of Fluorescent Patterns and Histochemistry.—Application of the direct fluorescent conjugate of IgG from serum L.H. to rat tissues confirmed

the staining pattern previously obtained with the double layer technique using PBC sera and anti- γ or anti- β_{1C} conjugates (4, 5). Almost all tissues have been examined and the detailed results will be presented elsewhere. Histochemical staining for succinic dehydrogenase on adjacent sections of each tissue gave almost identical patterns of similar intensity to those obtained with immunofluorescence (Figs. 1 and 2). The fluorescence in different cells was either diffuse or granular. Diffuse cytoplasmic staining was always bright, whereas in cells giving the granular pattern, the intensity was either bright, dull, or faint according to the tissue examined. In some tissues no fluorescence was visible. Characteristic examples for bright uniform fluorescence were salivary gland



TEXT-FIG. 5. Distribution of CF antigen and marker enzymes in mitochondrial and lysosomal fractions separated by centrifugation in a sucrose density gradient, using the livers of rats pretreated with Triton.

ducts, renal distal tubules, and gastric parietal cells. Striated and cardiac muscle were typical for the bright granular pattern, while gastric chief cells and proximal tubules gave dull granular fluorescence; liver cells stained only faintly and thymus and spleen were negative. The cytoplasmic fluorescence could be absorbed out with mitochondrial fractions using either the standard serum or other PBC sera.

Comparison of Fluorescence and CF Antigen Activity in Different Organs.—The antigenic activity in different organs was proportional to the intensity of immunofluorescent staining (Table I). Kidney and heart which showed bright fluorescence also had the highest antigen content, approximately 5000 units/g of tissue. Liver stained only faintly and contained less than half the amount of antigen, while thymus and spleen which showed no fluorescence had little antigenic activity. A similar relationship was evident when antigenic activity was

expressed in terms of tissue protein. The mitochondrial specific antigenic activity of heart and kidney were twice as high as that of the liver and a similar relationship held for the proportion of membrane-bound proteins between heart and liver. Since succinic dehydrogenase is a membrane-bound enzyme the close correlation in the intensity and distribution of fluorescent and histochemical staining suggested that the antigen was associated with the membranes. The following experiments were designed to investigate this further.

TABLE I
Comparison of Antigenic Activity in Different Organs Assessed by Reaction with Standard Primary Biliary Cirrhosis Serum

	Kidney	Heart	Liver	Spleen	Thymus
Fluorescence	+++	+++	+	—	—
Total CF antigen					
Units/g tissue	4800	5100	2150	750	700
Units/mg protein	26	25	11	3.6	3.5
Mitochondrial activity					
Units/mg protein	79	68	35	10	9
Membrane-bound protein		82%*	40%*		

* Data taken from Green (31).

Association of Complement-Fixing Antigen with Mitochondrial Membranes

Effect of Mitochondrial Fragmentation.—Mitochondria were fragmented with ultrasound, hypotonic solutions, detergents and solvents. Representative examples of these treatments are shown on Table II. Hypotonic phosphate buffer was the mildest treatment in that it caused no loss of antigenic activity whereas ultrasound, lysolecithin, and hexane reduced the total amount of antigen by 30–60%. The degree of mitochondrial fragmentation could be gauged from the sedimentation behavior of the antigen after treatment. Whereas all the antigen in the buffer control was sedimented by 0.4×10^6 g min, with hypotonic saline and hexane 18–25% still remained in the supernatant, but could be spun down by 12×10^6 g min. A greater degree of fragmentation was observed after sonication when 55% of the antigen sedimented in the first pellet while 18% was still present in the supernatant from the second pellet. Lysolecithin caused extensive breakdown to medium-sized fragments and only 14% of the antigen was sedimented at 0.4×10^6 g min; as much as 66% was recovered in the 12×10^6 g min pellet, leaving 20% as minute fragments in the final supernatant. Complete removal of CF activity in supernatant fractions could be obtained after passage on columns of Sephadex G75, G100, and G200, or by overnight

centrifugation at 150,000 g. The release and removal of soluble mitochondrial proteins by these treatments led to a pronounced increase in antigenic specific activity. The highest CF activity was found in the 12×10^6 g min hypotonic pellet and in the 0.4×10^6 g min lysolecithin pellet. This detergent pellet is of special interest since under these conditions contamination with soluble proteins would be minimal. With the milder hypotonic treatment there was a progressive increase in specific activity in the two pellets. A high speed pellet obtained similarly was examined by electron microscopy (Fig. 4). It contained numerous membrane vesicles as well as amorphous material. The presence of dense matrix granules suggests that the osmotic lysis had broken up some inner membranes.

TABLE II
Analysis by Differential Centrifugation of Mitochondria Fragmented by Different Treatments

Treatment	Per cent of total antigen recovered			Specific activity (antigen units per mg protein)			Loss of antigen (per cent of control)
	Pellet 0.4×10^6 g min	Pellet 12×10^6 g min	Super-natant	Pellet 0.4×10^6 g min	Pellet 12×10^6 g min	Super-natant	
10 mM PO_4 buffer	79	18	3	74	99	9	0
Hexane	74	25	<1	36	52	<1	46
Ultrasound	55	27	18	50	45	8	59
Lysolecithin	14	66	20	92	52	13	32
CFT buffer (control)	100	<1	<1	43	<4	<1	0

After treatment the mitochondrial suspension was spun at 18,000 rpm for 15 min and the supernatant centrifuged at 50,000 rpm for a further 80 min. The two pellets and final supernatant obtained after each treatment were analyzed for antigen content by quantitative complement fixation.

DISCUSSION

The antigen reacting with the sera of primary biliary cirrhosis patients was recovered predominantly in the mitochondrial fraction of tissue homogenates obtained by differential centrifugation. Studies of enzymes characteristic for mitochondria, lysosomes, and microsomes confirmed the association with mitochondria in that a high degree of correlation was found in all subcellular fractions between antigenic activity and succinic dehydrogenase, an enzyme known to be confined to mitochondrial inner membranes (16, 17, 18). There was poor correlation with the lysosomal and microsomal enzyme markers. Furthermore, purified preparations of lysosomes were devoid of antigen whereas a preparation of mitochondria shown to be virtually homogeneous by electron microscopy had the highest activity of any fraction isolated. In accord with these studies, immunofluorescent staining obtained in various tissues with the direct conjugate of a patient's serum, was comparable in intensity and distribution with the

pattern observed using the histochemical nitro-blue-tetrazolium method for demonstrating succinic dehydrogenase in mitochondria.

More precise localization of the antigen within the mitochondria was also undertaken. Essentially, these structures consist of an outer membrane, a soluble protein matrix, and an inner membrane with villous infoldings forming cristae which are associated with electron transport and oxidative phosphorylation. Mitochondria display considerable variations in size and shape according to the energy requirements in different tissues. Cells involved in active transport, e.g., renal tubules or gastric parietal cells, and those with contractile function, e.g., cardiac muscle, show an extensive development of cristae (19). The cells which gave intense staining with the fluorescent conjugate of PBC serum and which showed a high content of succinic dehydrogenase are all known to have mitochondria with closely packed cristae. The localization of the antigen on the cristae suggested by these histological findings would explain the high antigenic specific activity obtained with the complement fixation method when testing extracts of kidney and heart. On the other hand, liver mitochondria have less well developed cristae, stain faintly in the fluorescent and histochemical tests, and also have a lower antigenic specific activity in the CFT. The relation between the proportion of membrane-bound protein and the specific antigenic activity in different organ extracts would also favor an association of the antigen with membrane structures. Membrane pellets obtained after almost complete mitochondrial disruption had an increased specific antigenic activity whereas the supernatants containing soluble or easily detachable proteins released by the treatments had little antigenic activity. Thus repeated exposure of liver mitochondria to hypotonic solution, known to release up to 50% of the total proteins into the supernatant, did not diminish the antigenic activity of the ghost pellet. Furthermore, although osmotic lysis releases outer membrane fragments (18), the specific activity of the pellet consisting largely of inner membrane, was increased. Thus the experiments reported here not only favor the membrane-bound character of the PBC antigen but strongly suggest its association with the system of inner membranes cristae. Separation of the two mitochondrial membranes, verified by electron microscopy, has now shown that the antigen is associated almost exclusively with the inner membranes (20).

The present work in no way clarifies the question of why the destruction of bile duct cells characteristic of primary biliary cirrhosis (21-23) should be associated with the presence of mitochondrial antibodies. It is possible that these antibodies arise as an epiphenomenon, although it is difficult to see why mitochondrial antibodies of high titer should be so prevalent in this particular disease, while they were rarely found in other liver disorders associated with extensive tissue damage, or in mixed hospital patients and normal controls (5, 24). Bile stasis cannot be responsible since mitochondrial antibodies are notably absent in cases of jaundice due to extrahepatic obstructive lesions and in patients with drug jaundice having biochemical indications of biliary obstruction.

The essential lesion of primary biliary cirrhosis may be mediated by bile duct antibodies (25-27) or by cell-mediated hypersensitivity mechanisms. However, since the bile duct antibodies were found in over 50% of patients with other liver diseases having no connection with PBC and in 12% of normal individuals, their role in the pathogenesis of this disease remains uncertain.

In some instances autoantibodies are formed in response to infection by pathogenic organisms which share antigenic determinants with tissue components. This has been suggested in the case of ulcerative colitis (28), in rheumatic heart disease (29), and in certain cases of nephritis (30). It is of particular interest that syphilitic infection gives rise to antibodies which react in the Wasserman test with cardiolipin, a phospholipid component of mitochondrial inner membranes (18). Possibly this represents a cross-reaction with a lipid of the respiratory system in the treponema organism. The mitochondrial antigen reacting with PBC sera is distinct from cardiolipin as evidenced by the negative Wasserman reactions obtained in these patients; nevertheless, further study of the relationship of the mitochondrial antibodies to membrane components of different microorganisms may throw some light on the etiology of primary biliary cirrhosis.

SUMMARY

The antigen reacting with complement-fixing antibodies in the sera of patients with primary biliary cirrhosis was localized predominantly in the mitochondrial fraction of tissue homogenates obtained by differential centrifugation. Purified mitochondrial preparations had a high content of the antigen whereas purified lysosomes failed to fix complement with PBC sera. Analysis of a number of fractionation experiments showed a high correlation between antigen content and the mitochondrial enzyme succinic dehydrogenase in all fractions. There was much poorer correlation with lysosomal and microsomal enzyme markers. The patterns of staining obtained with a fluorescein conjugate of IgG from a PBC patient closely paralleled those obtained with a histochemical method for the demonstration of succinic dehydrogenase, further confirming the mitochondrial localization of the antigen. Staining was brightest in cells containing mitochondria with well-developed cristae. Studies on mitochondria fragmented by osmotic lysis, hexane, lysolecithin, and ultrasound suggest that the antigen is associated with the mitochondrial inner membranes.

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EXPLANATION OF PLATES

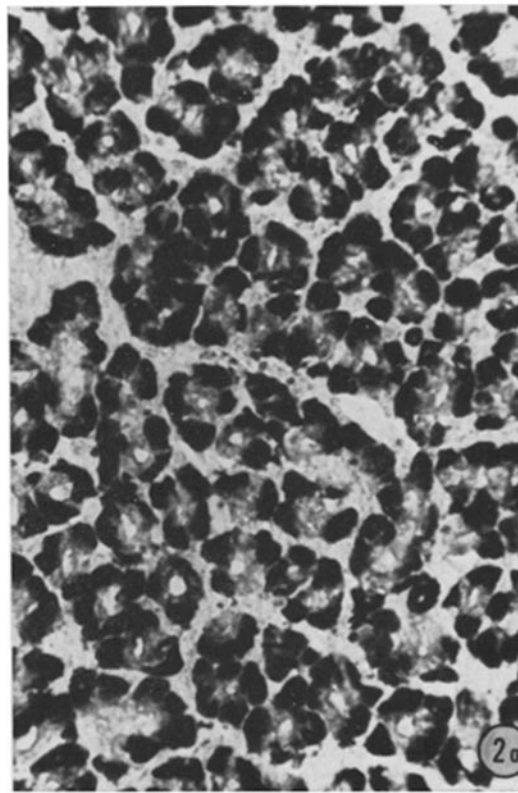
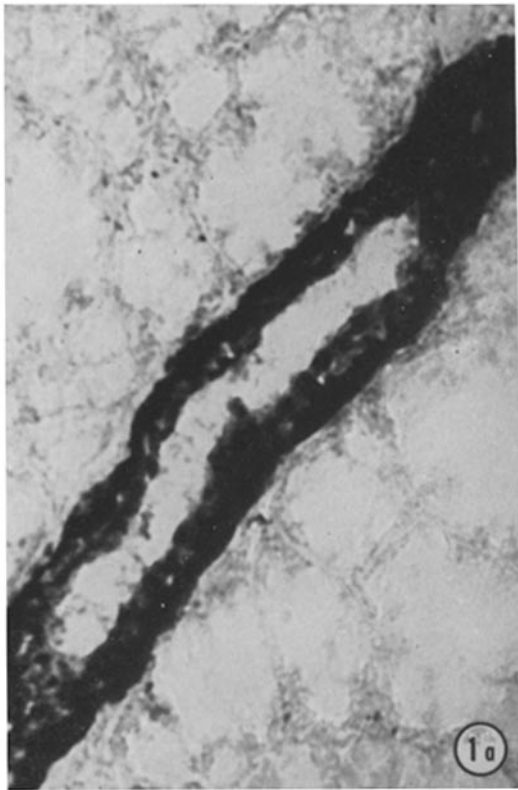
PLATE 26

FIG. 1 *a*. Section of rat submaxillary gland stained by the nitro-blue-tetrazolium (NBT) method for succinic dehydrogenase. $\times 350$.

FIG. 1 *b*. Serial section stained with a direct conjugate of primary biliary cirrhosis IgG. Both reagents gave diffuse cytoplasmic staining of the small salivary ducts and a faint granular pattern in acinar cells. $\times 350$.

FIG. 2 *a*. Section of rat gastric mucosa stained for succinic dehydrogenase. $\times 280$.

FIG. 2 *b*. Serial section stained with PBC conjugate. Both methods gave diffuse cytoplasmic staining of the parietal cells and a faint granular reaction with the chief cells. $\times 280$.



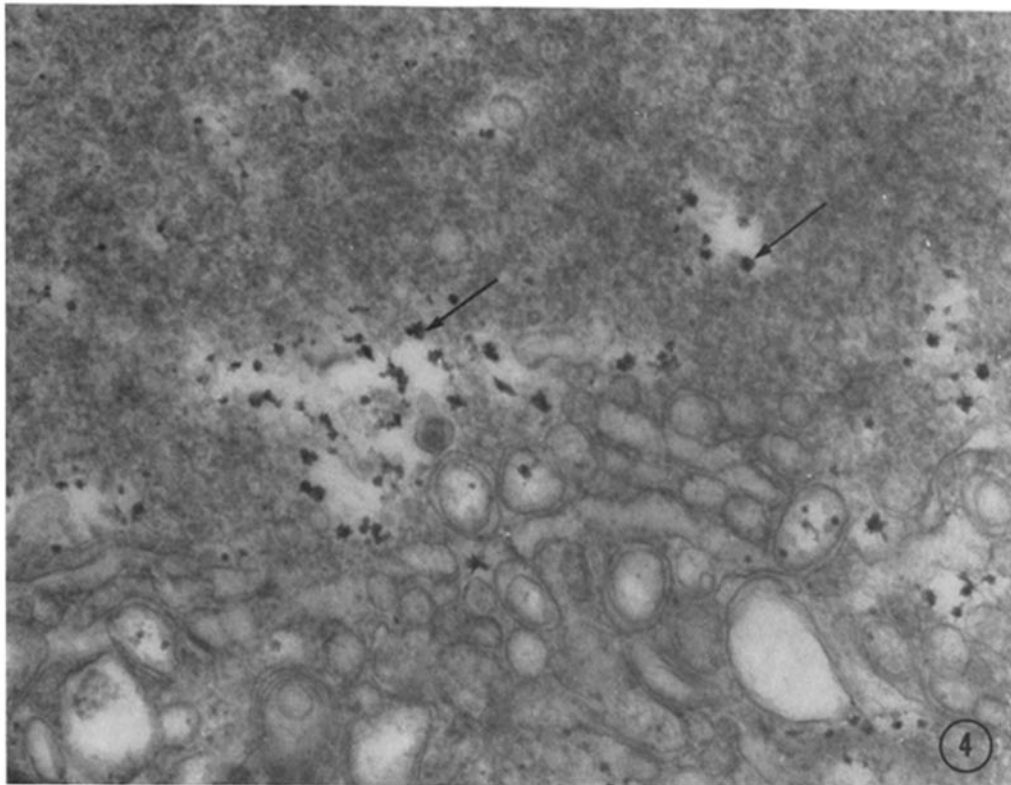
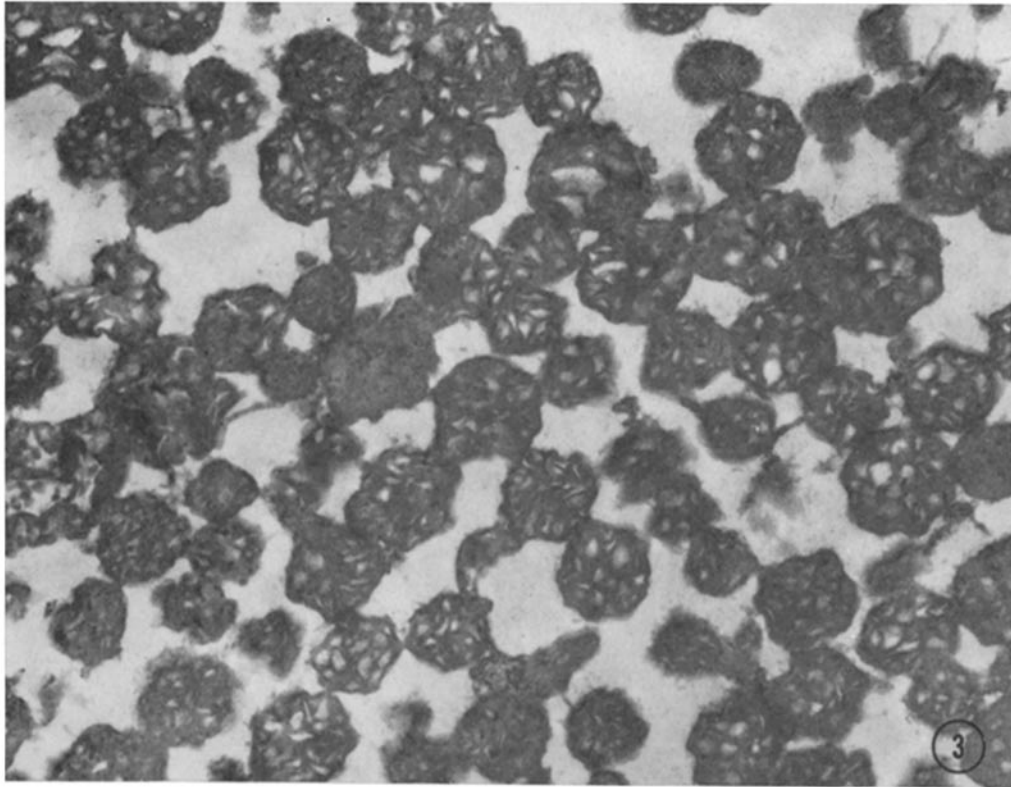
(Berg et al.: Mitochondrial antibodies in biliary cirrhosis)

PLATE 27

FIGS. 3 and 4. Electron micrographs of thin sections of material fixed in glutaraldehyde, treated with osmium tetroxide, and embedded in epoxy resin. The sections were stained with uranyl acetate.

FIG. 3. Mitochondrial fraction having a high specific activity for CF antigen. $\times 20,000$.

FIG. 4. Fragments released from mitochondria by osmotic lysis. The pellet showed membranous vesicles and amorphous material; free mitochondrial dense granules are also present (arrows) and indicate disruption of inner membranes. $\times 46,000$.



(Berg et al.: Mitochondrial antibodies in biliary cirrhosis)