CHARACTERIZATION OF RAT LIVER SUBCELLULAR MEMBRANES

Demonstration of Membrane-Specific Autoantigens

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ABSTRACT

The induction of acute hepatocellular necrosis in rats resulted in the production of complement fixing, IgM autoantibodies directed toward inner and outer mitochondrial membranes, microsomal membrane, lysosomal membrane, nuclear membrane, cytosol, but not to plasma membrane. Utilizing selective absorption procedures it was demonstrated that each subcellular membrane fraction possessed unique autoantigenic activity with little or no cross-reactivity between the various membrane fractions. It is proposed that the development of membrane-specific autoantibodies may provide an immunological marker useful in the differential characterization of various subcellular membranes.

INTRODUCTION

Elucidation of the complex structure and function of biological membranes has necessitated the use of a variety of experimental techniques. An approach which has been widely utilized to characterize cellular membrane systems is the localization of enzymatic activities resident on the individual membranes. As a result, certain enzymes have become established as specific markers for various subcellular membranes, e.g. cytochrome oxidase and succinate dehydrogenase for inner mitochondrial membrane (1), kynurenine hydroxylase and monoamine oxidase for outer mitochondrial membrane (1, 2), glucose-6-phosphatase for microsomal membrane (3), acid phosphatase for lysosomal membrane (4), and Mg^{++}-ATPase for plasma membrane (5). Assays for these marker enzymes are important experimentally for assessing the relative purity of membrane preparations; however, the usefulness of the technique may be limited since certain enzyme markers may not reside solely on one membrane but may be more widely distributed (6, 7). In addition, enzymatic activities resident on certain membranes may be abolished due to denaturation or to solubilization of the enzymes during tissue fractionation and subsequent storage of the membranes.

Another experimental approach which has been employed to characterize membrane systems is the localization of specific antigens on cellular and subcellular membranes. Although evidence has been reported regarding the localization of antigenic constituents on cellular membranes (8–14), most of these studies have employed antisera produced in heterologous animals. Since histocompatibility antigens are present on most cellular and subcellular membranes (15, 16), heterologous antisera would contain antibodies directed toward common histocompatibility antigens as well as po-

tential organelle-specific antigens. The use of autoantibodies could overcome such difficulties as it has been demonstrated that immunological tolerance does not exist with respect to autologous subcellular membranes (17). Hence, after the induction of acute liver necrosis in rats (18, 19), autoantibodies are produced which react with various subcellular fractions and especially with antigens on the mitochondrial fraction of the rat liver (20). Such autoantibodies directed toward subcellular membranes should be more specific than heterologous antiserum, since they should not contain antibodies directed toward the histocompatibility antigens. Indeed, using autoantibodies induced by the subcutaneous injection of carbon tetrachloride, we have demonstrated that inner and outer mitochondrial membranes possess unique autoantigenic determinants (21).

The present study was designed to determine whether autoantibodies to all subcellular membranes are produced after acute liver necrosis and if these autoantibodies react with unique autoantigenic determinants on each membrane. These studies could be an important experimental method for the identification and characterization of subcellular membranes independent of either the assay of enzymatic markers or of the use of heterologous antisera to membrane consitutents.

METHODS

Isolation of Subcellular Constituents

Mitochondria were isolated from rat liver and were separated into their inner and outer membranes using a modification of the procedure described by Parsons et al. (22). All procedures were performed at 0°–4°C. Eight to ten adult Wistar rats were stunned and sacrificed by decapitation. A 1:10 (wt/vol liver homogenate was prepared using 0.25 M sucrose and was centrifuged at 480 *g* for 10 min. The supernate was removed and was centrifuged at 5,090 *g* for 20 min. After removal of the fatty surface layer, the supernate was decanted and was used for the isolation of the microsomal fraction and the cytoplasm. The pellet, containing whole mitochondria, was washed twice by resuspending the mitochondria in 15 ml of 0.25 M sucrose and centrifuging the suspension at 7,700 *g* for 10 min. Inner and outer mitochondrial membranes were separated by swelling isolated whole mitochondria from six rat livers in 500 ml of 20 mM potassium phosphate buffer (swelling buffer), pH 7.2, for 20 min with gentle stirring. The suspension was centrifuged at 34,800 *g* for 20 min. The supernate was discarded and the pellet was resuspended in 300 ml of the swelling buffer and was centrifuged at 1,935 *g* for 15 min to sediment the inner membrane. The supernate, containing crude outer membrane, was decanted carefully and was centrifuged at 27,000 *g* for 30 min to sediment the outer membrane. Inner mitochondrial membrane was washed twice by resuspending the membrane in 20 ml of the swelling buffer followed by centrifuging at 3,020 *g* for 15 min. The inner membrane was suspended in 0.25 M sucrose. Outer mitochondrial membrane was resuspended in 15 ml of the swelling buffer and 5 ml of the suspension were layered on each of three discontinuous sucrose gradients prepared as previously described (22). The gradients were centrifuged at 56,000 *g* for 90 min. The crude outer membrane fraction separated into three bands on the gradient. Only the outer membrane fraction appearing in the most dense of the three gradient bands was used in this study as this fraction contained the highest specific activities of the outer membrane enzyme markers of the three bands taken from the gradient. The outer membrane was resuspended in 25 ml of 0.25 M sucrose and was centrifuged at 34,800 *g* for 45 min. The purified outer membrane was resuspended in 0.25 M sucrose.

Microsomal membrane and cytoplasm were isolated from the supernate obtained during the isolation of whole mitochondria. The supernate was centrifuged at 25,000 *g* for 20 min. The pellet was discarded and the supernate was centrifuged at 80,800 *g* for 90 min to sediment the microsomal membrane; the membrane was resuspended in 0.25 M sucrose. The supernate from this centrifugation containing the cytoplasm, also was collected.

Plasma membrane and nuclear membrane were isolated as described by Ray (23) and by Blobel and Potter (24), respectively. Lysosomes were isolated according to the procedure of Sawant et al. (25). The purified lysosomes were resuspended in 30 ml of 0.7 M sucrose and were sonicated at 75 W for 30 s. The suspension was centrifuged at 34,500 *g* for 30 min to sediment the lysosomal membrane. The subcellular membranes prepared using the procedures outlined above were suspended in 0.25 M sucrose and were stored frozen at −20°C. The protein concentration of each subcellular constituent was estimated using a micro-Kjeldahl procedure (26), assuming a protein nitrogen content of 16%.

Enzyme Analyses

The relative purities of the isolated subcellular constituents were assessed by comparing the specific activities of the following marker enzymes in each preparation: cytochrome oxidase (1), succinate dehydrogenase (1), succinate-cytochrome *c* reductase (27), monoamine oxidase (28), kynurenine hydroxylase (1) rotenone-insensitive NADH-cyto-

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chrome *c* reductase (27), Mg^{++}-ATPase (29), and acid phosphatase (30). Glucose-6-phosphatase was assayed as described by Swanson (31) and inorganic phosphate was estimated by the method of Gomori (32).

Development of Anti-Liver Autoantibodies

Acute hepatocellular necrosis was induced in 132 adult Wistar rats in order to produce autoantibodies to autologous subcellular membrane. Three procedures were utilized to induce acute liver damage: (*a*) the subcutaneous injection of 0.03 ml of carbon tetrachloride per 100 g of body weight (33); (*b*) the administration by gavage of 3.0 ml of 9.5 mM dimethylnitrosamine (34); and (*c*) freezing a portion of the left lobe of the liver. Freezing was accomplished by exposing the liver through a midline abdominal incision and touching the left lobe of the liver with a 2.0 × 4.0 cm stainless steel cylinder filled with liquid nitrogen. Ten additional rats served as controls and were not subjected to the induction of hepatocellular damage; these rats were used to assess whether multiple cardiac punctures might induce the development of autoantibodies which cross-reacted with liver subcellular constituents. Both experimental and control rats were divided into two groups; the first group was bled by cardiac puncture before and 1, 3, 5, 7, and 9 days after the induction of acute liver damage; the second group was bled before and 2, 4, 6, 8, and 10 days after the induction of hepatocellular necrosis. All cardiac punctures were performed on rats lightly anesthetized with ether. The amount of serum glutamic-oxalacetic transaminase (SGOT) in each serum sample was assayed as described by Bergmeyer (35). All serum samples were stored at −20°C.

Complement Fixation Tests

Complement fixation tests were performed using a microtiter apparatus (Cooke Laboratory Products, Cooke Engineering Co., Alexandria, Va.) as described by Pinckard et al. (36). All serum samples to be tested were heat inactivated at 56°C for 30 min to inactivate rat C1, C2, C3, and C4 (37). Doubling dilutions of sera were made in sodium barbital-buffered saline, pH 7.5, containing 0.1% bovine serum albumin (26). After the addition of 1.5-minimal hemolytic doses of guinea pig complement, an optimal concentration of a subcellular constituent was added, as determined by a checkerboard titration. The microtiter plate was incubated at 37°C for 30 min. Sensitized sheep erythrocytes were added, and the microtiter plate was incubated at 37°C for 40 min. The plate was centrifuged at 450 *g* for 5 min. The end point of the titration was defined as the highest serum dilution at which there were clearly visible sheep erythrocytes.

Absorption Procedure

Absorption of 0.3 ml of a pool of heat-inactivated sera with increasing protein concentrations of various subcellular constituents was performed at 0°C. The serum samples were incubated for 2 h with amounts of subcellular constituents ranging from 1.5 to 300 μg protein. Each suspension was centrifuged at 15,000 *g* for 90 min at 4°C. The supernate was removed and was tested for autoantibody activity using the complement fixation test.

Immunoglobulin Nature of the Anti-Liver Complement-Fixing Activity

The immunoglobulin nature of the anti-liver complement-fixing activity of heat-inactivated sera from rats which had been injected with carbon tetrachloride was assessed using affinity chromatography (38). The gamma globulin fractions of sera from two rabbits immunized with purified rat light chains were isolated by sodium sulfate precipitation; in addition, the gamma globulin fraction of normal rabbit serum was isolated to serve as a control. 10-mg amounts of each gamma globulin fraction were mixed with 7 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) The Sepharose was washed with sodium barbital-buffered saline, pH 7.5, diluted to a total volume of 10 ml, and poured into a column. Heat-inactivated sera from rats possessing detectable complement-fixing activity with subcellular components were centrifuged on 24 sucrose gradients prepared as previously described (21); gradient fractions 6–10 containing all the complement-fixing activity were pooled and were concentrated by negative pressure dialysis. 2 ml of the resulting solution were applied to each Sepharose 4B column; 2-ml fractions were collected. Each fraction was tested for anti-mitochondria autoantibody activity using the complement fixation test.

As can be seen in Fig. 1, no complement-fixing activity to liver mitochondria was detected in the effluent from the column prepared with anti-rat light chain antiserum, while autoantibody activity was detected in fractions 2 and 3 from the column prepared with normal rabbit serum. These data demonstrated the immunoglobulin nature of the anti-liver mitochondria complement-fixing activity by indicating the presence of light chains. Additional information indicating the immunoglobulin class of the anti-liver complement-fixing activity was obtained using sucrose density gradient ultracentrifugation. The complement-fixing activity sedimented as a

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19S component, typical of the IgM class of immunoglobulins.

RESULTS

Purification of Subcellular Constituents

In order to localize autoantigens on various subcellular membranes, whole mitochondria, inner and outer mitochondrial membranes, microsomal membrane, cytoplasm, plasma membrane, lysosomal membrane, and nuclear membrane were isolated from the livers of normal rats. Analyses for various marker enzymes were performed using each subcellular fraction to estimate the relative purity of each of these preparations. The results of these analyses are shown in Tables I and II. Using the specific activities of the various marker enzymes as an indication of the presence of a given subcellular membrane component in the membrane preparations, each of the individual preparations showed a significant enrichment of the marker enzymes characteristic of the desired subcellular component. In general, cross contamination of the various preparations of subcellular constituents was minimal. The results of these fractionation procedures were essentially comparable to those obtained by the authors who originated the individual membrane preparations cited above, in the methods section. As might be expected due to the fractionation techniques employed, the lysosomal and nuclear membrane preparations contained some mitochondrial membrane markers, while the microsomal membrane preparation contained some outer mitochondrial membrane depending upon which outer membrane marker was considered as the indicator. The effects of significant cross contamination of the individual membrane preparations on the results of the present study will be discussed later when the anti-mem-

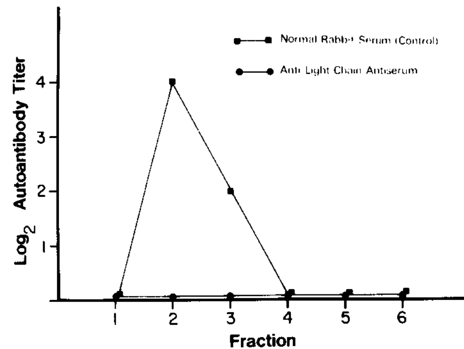

FIGURE 1 Affinity chromatography of heat-inactivated rat sera on Sepharose 4B columns. Procedures followed in this experiment are described in the Methods section.

TABLE I

Relative Specific Activities (Nanomoles/Minute per Milligram Protein) of Marker Enzymes in the Whole Mitochondria, Inner and Outer Mitochondrial Membranes, and Microsomal Membrane Preparations

		Whole mitochondria	Inner membrane	Outer membrane	Microsomal membrane
Inner mitochondrial membrane markers	Cytochrome oxidase	821.7	1,358.9	41.1	5.1
	Succinate dehydrogenase	156.3	247.8	96.0	17.0
	Succinate-cytochrome *c* reductase	26.9	64.0	7.3	1.4
Outer mitochondrial membrane markers	Monoamine oxidase	0.63	0.32	9.28	0.20
	Kynurenine hydroxylase	0.81×10^{-3}	0.56×10^{-3}	14.69×10^{-3}	0.06×10^{-3}
	NADH-cytochrome *c* reductase (rotenone insensitive)	197.1	81.7	4,120.9	784.8
Plasma membrane marker	Mg^{++}-ATPase	11.5	35.9	0.0	13.6
Microsomal membrane marker	Glucose-6-phosphatase	4.52	2.60	41.6	103.2
Lysosomal membrane marker	Acid phosphatase	29.0	13.2	228.7	37.0

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TABLE II

Relative Specific Activities (Nanomoles/Minute per Milligram Protein) of Marker Enzymes in the Cytoplasm Plasma Membrane, Lysosomal Membranes, and Nuclear Membrane Preparations

		Cytoplasm	Plasma membrane	Lysosomal membrane	Nuclear membrane
Inner mitochondrial membrane markers	Cytochrome oxidase	0.0	0.0	22.6	92.9
	Succinate dehydrogenase	15.8	54.1	332.1	216.8
	Succinate-cytochrome *c* reductase	0.0	6.5	36.4	16.5
Outer mitochondrial membrane markers	Monoamine oxidase	0.0	0.18	0.0	0.28
	Kynurenine hydroxylase	0.26×10^{-3}	0.0	3.02×10^{-3}	1.37×10^{-3}
	NADH-cytochrome *c* reductase (rotenone insensitive)	17.3	13.2	194.4	141.0
Plasma membrane marker	Mg^{++}-ATPase	1.3	209.6	0.0	21.3
Microsomal membrane marker	Glucose-6-phosphatase	0.0	13.4	0.0	0.0
Lysosomal membrane marker	Acid phosphatase	20.6	26.3	296.6	67.7

brane autoantibody absorption experiments are described.

Incidence and Temporal Development of Autoantibodies

The development of autoantibodies directed toward liver subcellular components was effected in three groups of rats by experimentally inducing acute hepatocellular necrosis. Serum samples obtained from each group of rats were tested for autoantibody activity using a complement fixation test and the eight subcellular membrane preparations as test antigens. The protein concentration of each subcellular component necessary to achieve a mzximum complement fixation titer was determined from checkerboard titrations using sera known to contain autoantibody when tested against crude rat liver homogenates as the test antigen. As shown in Table III, the minimum amount of the various subcellular membranes required to give a maximum complement fixation reaction varied considerably. It is of major significance that no autoantigenic activity could be detected when plasma membrane was used as the test antigen even at protein concentrations as high as 4 mg/ml.

TABLE III

Relative Protein Concentrations (Micrograms/Milliliter) of Subcellular Components Required for Maximum Complement-Fixation Titers

Subcellular component	
	μg/ml
Whole mitochondria	440 ± 45
Inner mitochondrial membrane	510 ± 27
Outer mitochondrial membrane	80 ± 10
Microsomal membrane	350 ± 18
Cytoplasm	610 ± 73
Plasma membrane	Not antigenic*
Lysosomal membrane	180 ± 45
Nuclear membrane	240 ± 36

* Addition of plasma membrane in concentrations up to 4,000 μg/ml did not result in complement fixation in the test system.

The percentages of rats which developed detectable complement-fixing activity to each of the subcellular membranes after exposure to acute hepatocellular necrosis by carbon tetrachloride injection, by dimethylnitrosamine administration, or by freezing the liver are shown in Figs. 2, 3, and 4, respectively. An individual rat was considered

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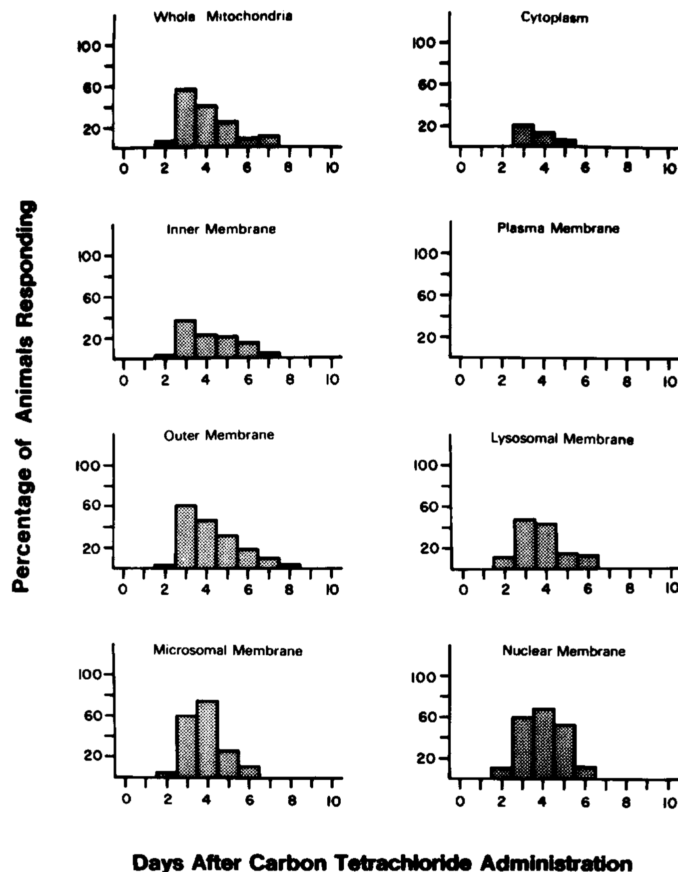

FIGURE 2 Percentage of rats which responded to various subcellular fractions using heat-inactivated sera obtained before and after carbontetrachloride administration on day 0. Experimental procedures are listed in the Methods section.

to have responded only if the complement-fixing titer was equal to or greater than eight. Autoantibody activity was not detected in serum samples obtained before the individual treatments on day 0. In each of the different treatments, autoantibody activity initially was detected on day 2; maximum numbers of rats developed autoantibodies to all subcellular membranes except plasma membrane between days 3 and 6 depending upon the method used to induce liver necrosis. Usually, after day 8, no complement-fixing activity was detected, except for the experiment in which the livers were frozen to produce the liver damage.

A suggestion that the autoantigens on the various subcellular membranes may be unique might be derived from the observation that the percentages of rats responding to the various test antigens was clearly different on any given day of the test period. This was the case for each of the three experiments irrespective of the method used to induce the hepatocellular necrosis.

The data obtained from the three groups of experimental rats offer a striking contrast to that obtained from the control rats. Control rats were bled by cardiac puncture on each day of the test period but were not subjected to the induction of hepatocellular necrosis. These rats failed to develop autoantibody activity to any test autoantigen on any day of the test period.

Concomitant with the increased percentages of animals possessing autoantibody after the induction of acute liver damage, an increase in the average autoantibody titers of those rats responding to the subcellular autoantigens also was observed. Figs. 5, 6, and 7 show the average $\log_2$ autoantibody responses of the rats in which acute liver damage was induced by the administration of carbon tetrachloride, dimethylnitrosamine, and

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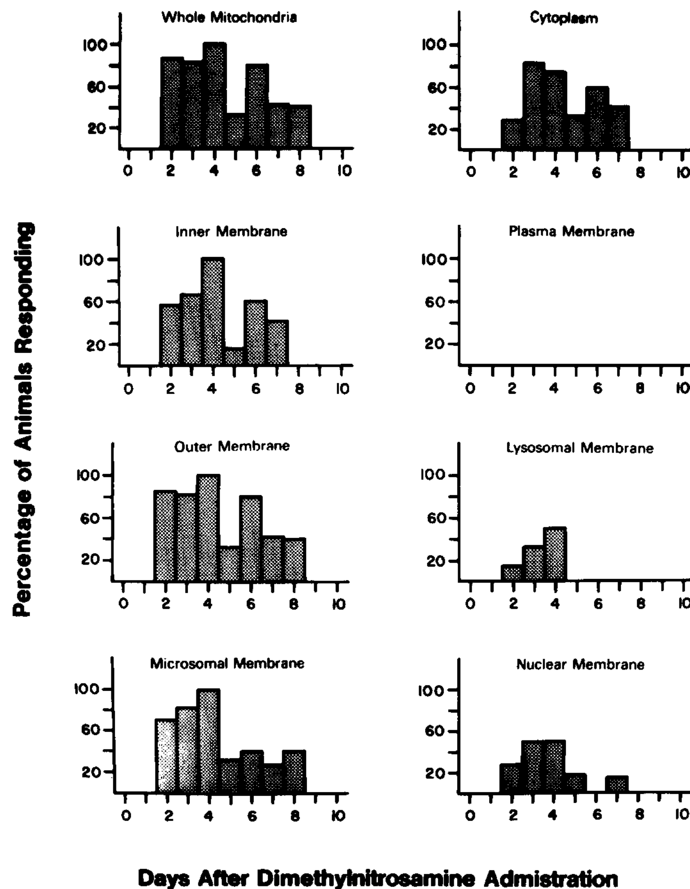

FIGURE 3 Percentage of rats which responded to various subcellular fractions using heat-inactivated sera obtained before and after dimethyl-nitrosamine administration on day 0.

by freezing a portion of the liver, respectively. In addition, the average number of units of SGOT activity measured on each day are shown for the three groups of experimental rats. Maximum autoantibody titers to most autoantigens in the rats treated with CCl_4 and dimethylnitrosamine were detected 3 or 4 days after the induction of liver necrosis on day 0. In the animals subjected to acute hepatic injury by freezing the liver, maximum autoantibody titers to most subcellular components were observed on days 5 and 6. The increased level of SGOT preceded increased autoantibody activity by 24 h in the three groups of experimental rats. Again, a suggestion as to the uniqueness of the autoantibodies developed toward the individual subcellular membranes may be taken from the fact that the average autoantibody titer of the rats responding to hepatocellular necrosis was significantly different for each of the subcellular membranes.

Characterization of Autoantigenic Specificity of Subcellular Membranes

Determination of the autoantigenic specificities of the subcellular membranes was accomplished by absorbing a pool of sera from rats injected with CCl_4 with increasing protein concentrations of each subcellular membrane. Each absorbed serum sample was tested for autoantibody activity using the complement fixation test and various subcellular constituents as the test autoantigens. The results of the studies using inner mitochondrial membrane are shown in Fig. 8. Absorption with increasing protein concentrations of inner membrane resulted in a four $\log_2$ reduction in the autoantibody titer to inner membrane; the titers to cytoplasm and microsomal membrane remained unchanged while those to outer membrane, nuclear membrane, and lysosomal membrane were reduced by only a single $\log_2$ dilution.

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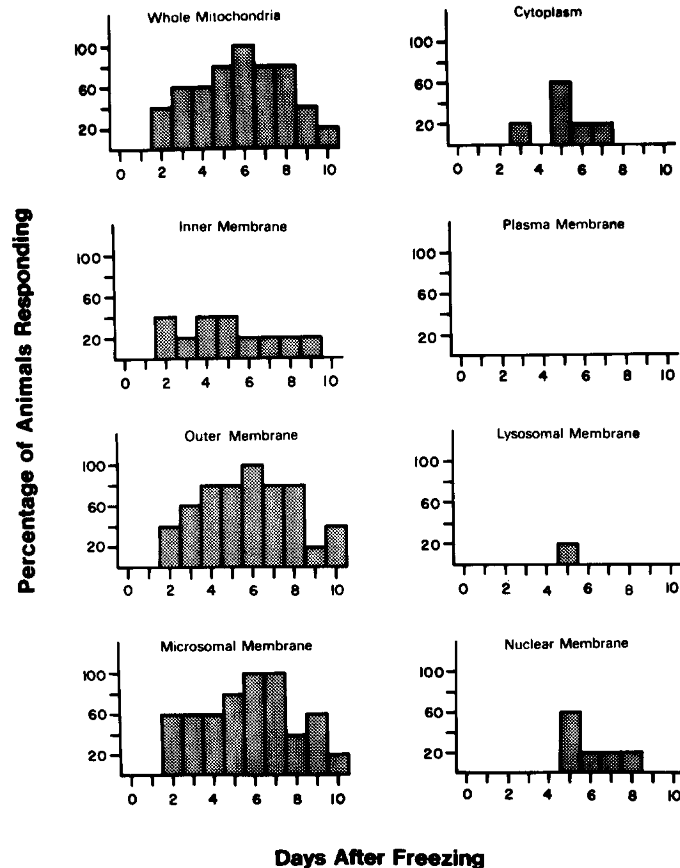

FIGURE 4 Percentage of rats which responded to various subcellular fractions using heat-inactivated sera obtained before and after induction of the liver injury by freezing on day 0.

The results of absorbing the same serum pool with increasing protein concentrations of outer membrane are shown in Fig. 9. Absorption with 0.5 mg of outer membrane per ml pooled serum resulted in a three $\log_2$ reduction in the autoantibody titer to outer membrane while the titers to the remaining autoantigens were unchanged or were reduced by only one $\log_2$ dilution.

Fig. 10 shows the results of absorbing the same pool of sera with increasing protein concentrations of microsomal membrane. A three $\log_2$ reduction in the autoantibody titer to microsomal membrane was noted after adsorption with 1.0 mg of the membrane per ml of pooled sera. Reductions of only one $\log_2$ dilution were noted in the titers to inner and outer mitochondrial membranes and cytoplasm; the titers to nuclear membrane and lysosomal membrane were unaffected by absorption with microsomal membrane.

The results of absorbing the same serum pool with increasing amounts of lysosomal membrane are shown in Fig. 11. All autoantibody activity to lysosomal membrane was removed after adsorption with 0.5 mg of the membrane; however, only a single $\log_2$ reduction was noted in the autoantibody titers to inner and outer mitochondrial membranes and nuclear membrane. The titers to microsomal membrane and cytoplasm were unchanged after absorption with lysosomal membrane.

Fig. 12 shows the results of absorbing the same pool of sera with increasing protein concentrations of nuclear membrane. Only one $\log_2$ reduction was noted in the autoantibody titers to inner and outer mitochondrial membranes, microsomal membrane, and lysosomal membrane while no reduction of autoantibody titer was observed to cytoplasm. The autoantibody titer to nuclear membrane was reduced three $\log_2$ dilutions and was not detected after absorption with the largest amount of the membrane.

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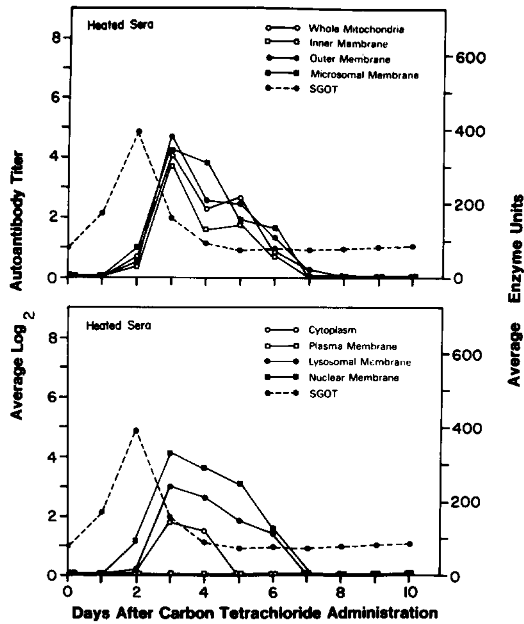

FIGURE 5 Average $\log_2$ autoantibody titers to various subcellular fractions and the SGOT response in heat-inactivated sera before and after carbontetrachloride administration on day 0.

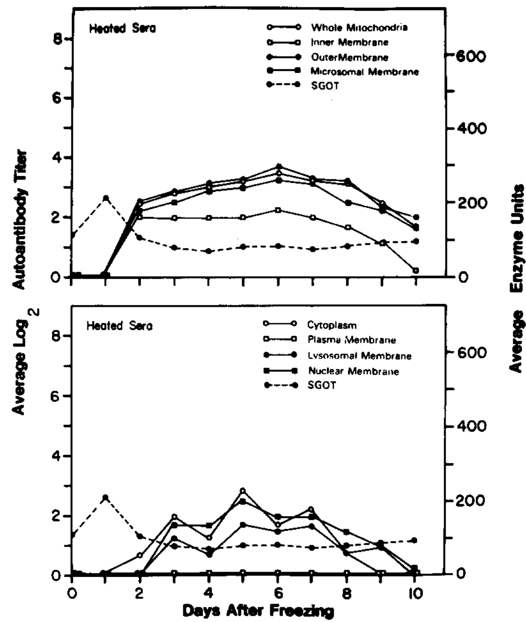

FIGURE 7 Average $\log_2$ autoantibody titers to various subcellular fractions and the SGOT response in heat-inactivated sera before and after the induction of liver damage by freezing on day 0.

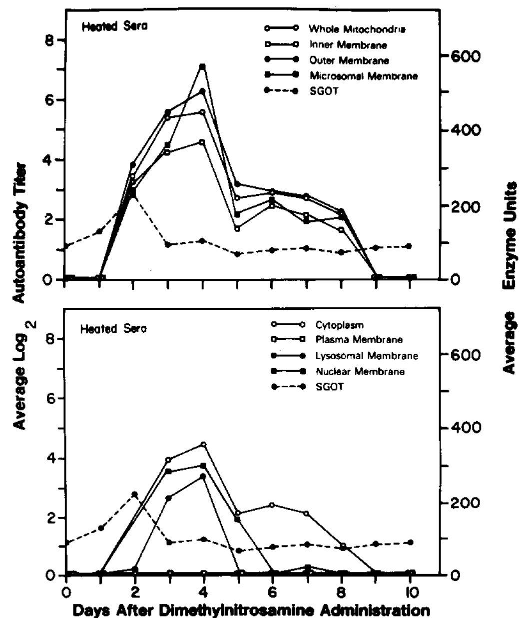

FIGURE 6 Average $\log_2$ autoantibody titers to various subcellular fractions and the SGOT response in heat-inactivated sera before and after dimethylnitrosamine administration on day 0.

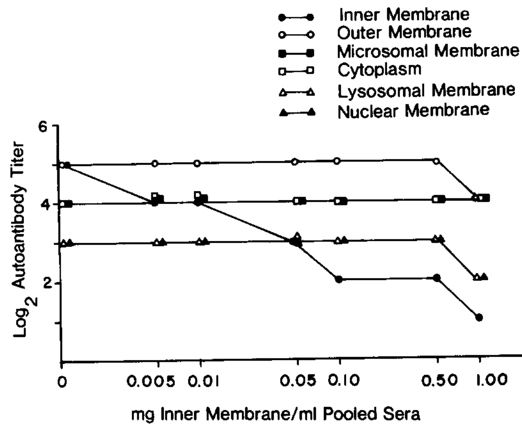

FIGURE 8 Log_2 autoantibody titer toward various subcellular fractions of a pool of heat-inactivated sera from carbon tetrachloride rats which has been absorbed with increasing amounts of rat liver inner mitochondrial membrane. Absorption procedures are described in the Methods section.

DISCUSSION

The development of tissue fractionation procedures by which preparations of various cellular and subcellular membranes may be obtained in relatively pure form has afforded an opportunity to compare

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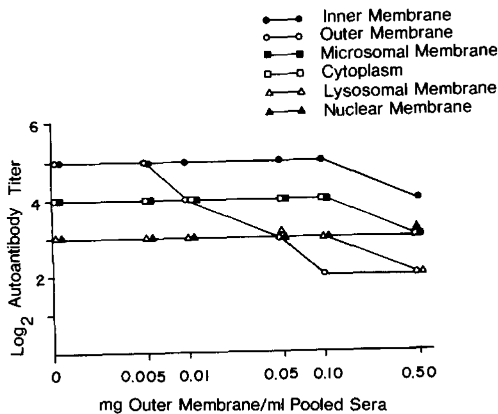

FIGURE 9 Log_2 autoantibody titer toward various subcellular fractions of a pool of heat-inactivated sera from carbon tetrachloride-treated rats which has been absorbed with increasing amounts of rat liver outer mitochondrial membrane.

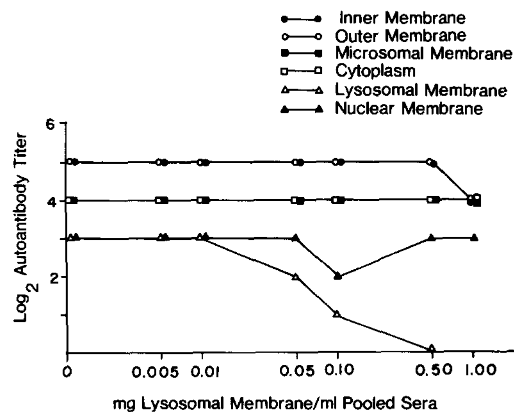

FIGURE 11 Log_2 autoantibody titers toward various subcellular fractions of a pool of heat-inactivated sera from carbon tetrachloride-treated rats which has been absorbed with increasing amounts of rat liver lysosomal membrane.

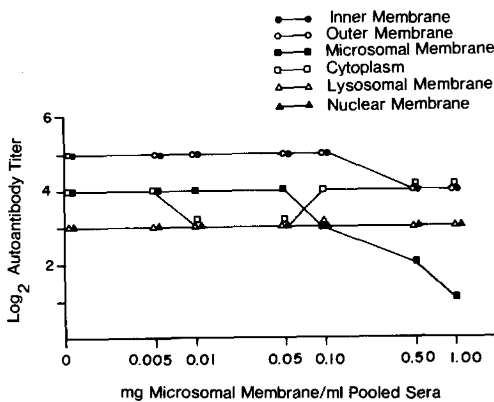

FIGURE 10 Log_2 autoantibody titers toward various subcellular fractions of a pool of heat-inactivated sera from carbon tetrachloride-treated rats which has been absorbed with increasing amounts of rat liver microsomal membrane.

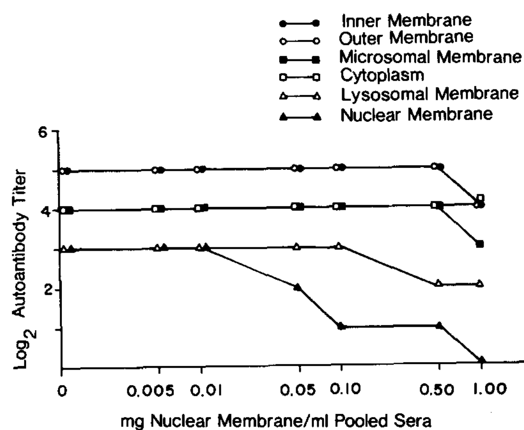

FIGURE 12 Log_2 autoantibody titers toward various subcellular fractions of a pool of heat-inactivated sera from carbon tetrachloride-treated rats which has been absorbed with increasing amounts of rat liver nuclear membrane.

and contrast these membranes using a variety of analytical techniques. Cataloging the wide diversity of enzymatic constituents in cellular membranes (39, 40), comparing various solubilized membrane proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis (41, 42), and analyzing the membrane lipid composition of various cellular membranes (40, 43) have been techniques instrumental in the elucidation of salient features of the subcellular membranes of the rat liver parenchymal cell. Although these studies have demonstrated that certain subcellular membranes possess selected components and/or functions indicating identity, the major conclusions drawn from most of these comparative studies seem to illustrate the nonidentity, in fact, the nearly complete uniqueness of the individual membranes of the liver cell. Those studies which have included comparisons of similar organelle-derived membranes prepared from different tissues have indicated even sharper dissimilarities between such membranous components.

Few, if any, recent studies have included a rigorous comparison of the immunological properties of the different subcellular membranes of the

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liver cell. Hence, the utility of the present study derives from the fact that it represents an attempt to define the autoantigenic specificities of the various subcellular membranes prepared from rat liver. The results of this study demonstrate that inner and outer mitochondrial membranes, microsomal membrane, lysosomal membrane, and nuclear membrane each possess unique autoantigenic determinants with no detectable cross-reaction between the various membranes. This conclusion is based on the results of absorption experiments (see Figs. 8–12) which indicate that absorption of pooled sera with increasing protein concentrations of each subcellular membrane reduced or eliminated the autoantibody activity to that membrane; however, the autoantibody titers to the remaining subcellular membranes were not reduced significantly. Throughout this study a single $\log_2$ increase or decrease in the complement-fixing titer was not considered significant because of the limitations inherent in the test procedure; these limitations derive from the use of doubling dilutions of sera, the use of mechanical diluters which may transfer slightly different amounts of sera, and from the evaluation of the test by visual inspection.

Although sera were not absorbed with cytoplasm each of the sera which was absorbed with the five subcellular membranes was tested for activity directed toward cytoplasm. Since significant reductions in the autoantibody titers to cytoplasm were not noted after any absorption procedure, it also is concluded that cytoplasm possesses autoantigenic determinants unique from those of the five subcellular membranes used in the absorption experiments. The nature of the cytoplasmic antigenic material is at present not known, but may be small membrane fragments which do not sediment at 100,000 *g*.

Elucidation of the autoantigenic specificities of subcellular constituents as accomplished in this study is dependent upon the relative purities of the subcellular components used in the absorption experiments. Estimation of the relative purities of these membrane preparations on the basis of enzymatic analyses is only valid if a particular enzyme can be localized solely to a single subcellular constituent. Several investigators have demonstrated that most of the marker enzymes shown in Tables I and II are specific for the membranes indicated (1–3, 25, 29, 44). One possible exception, however, is the rotenone-insensitive NADH-cytochrome *c* reductase which has been shown to exist on both outer mitochondrial membrane and microsomal membrane (45). The localization of this enzyme on both membranes would account for the relatively high enzymatic activity which was detected in the two membrane preparations used in the present study.

The relative specific activities of the inner membrane marker enzymes, especially succinate dehydrogenase and succinate-cytochrome *c* reductase, suggest that the preparations of both lysosomal membrane and nuclear membrane were contaminated with inner mitochondrial membrane. Furthermore, it would appear that the preparation of outer mitochondrial membrane was contaminated with lysosomal membrane. However, if the relative purities of these three membrane preparations were assessed using the results of the absorption experiments, it would seem that there was relatively little contamination between the membrane preparations in question. The apparent discrepancy between the enzymatic data and the results of the absorption experiments could be due to the presence of nonmembrane-bound enzymes in the various membrane preparations. Alternatively, the presence of enzyme molecules bound to membrane fragments which did not possess autoantigenic determinants reactive with autoantibodies in the serum pool also could result in detectable enzymatic activity without corresponding autoantigenic activity. Direct correlation of enzymatic data with the results of absorption procedures based on antigen-antibody interactions cannot be accomplished unless the antibodies were directed only toward exposed antigenic determinants on enzyme molecules. This is not considered to be likely and the observed inconsistencies between the enzymatic data and the results of the absorption experiments are thought to result from the use of procedures which measure two inherently different types of reactions.

The lack of autoantibody activity directed toward plasma membrane demonstrates that intrinsic immunologic tolerance exists toward that membrane. However, the lack of tolerance directed toward other subcellular membranes has been demonstrated (17). Assuming that both surfaces of plasma membrane are exposed to the lymphoreticular system after cell necrosis, the lack of autoantibody activity further suggests that immunologic tolerance exists to both the inner and outer surfaces of plasma membrane. Since antibodies directed toward plasma membrane can be produced in heterologous animals (5, 8, 13), the antibodies

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must be directed toward histocompatibility or other allo- or isoantigens on the membrane. Unless such antibodies are directed toward determinants uniquely localized on a single subcellular membrane, the antisera would not be useful for identifying or characterizing that membrane.

The results of the present study indicate that antiserum specific for any of the subcellular membranes used in this study except plasma membrane could be prepared. A suggested procedure for the preparation of such antisera would include: (*a*) induction of liver necrosis using either carbon tetrachloride or dimethylnitrosamine; (*b*) collection of serum 3 or 4 days later; (*c*) sequential absorption of the serum with purified subcellular membranes other than the one toward which the antiserum is to be directed. Such highly specific antiserum would be useful for assessing the purities of preparations of subcellular membranes, for elucidating the biochemical nature and distribution of unique membrane determinants on the surfaces of subcellular membranes, or for detecting subtle alterations in subcellular membrane structure in cells undergoing neoplastic transformation.

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